Technical Communication No. 1. October 1939.

IMPERIAL BUREAU OF DAIRY SCIENCE
SHINFIELD, NEAR READING, ENGLAND.

The Phosphatase Test for Control of Efficiency of Pasteurization

by

H. D. KAY, R. ASCHAFFENBURG AND F. K. NEAVE.

F8,3C:6;8a17
N39
503

Price 2/-

CFTRI-MYSORE
503
Phosphatase Test

Technical Communication No. 1. October 1939.

IMPERIAL BUREAU OF DAIRY SCIENCE
SHINFIELD, NEAR READING, ENGLAND.

The Phosphatase Test for Control of Efficiency of Pasteurization

by

H. D. KAY, R. ASCHAFFENBURG AND F. K. NEAVE.

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE, * MYSORE. * LIBRARY

Price 2/-

503

CONTENTS.

	Page
Introduction	3
Phosphatase	5
General	5
Milk phosphatase	5
Phosphatase Determination in Milk	7
General technique for phosphatase determination	7
Original method	9
The Kay-Graham test	9
Gilcreas and Davis' modification	12
Methods of colour reading	13
Scharer's modification	14
Other modifications	15
Sensitivity of the Phosphatase Test	16
(i) With regard to temperature of pasteurization	16
(ii) With regard to holding time	18
(iii) As regards the presence of raw in pasteurized milk	19
Effect of Storage, Bacterial Contamination, Etc. on the Reliability of the Phosphatase Test	21
Storage	21
Preservatives	22
Bacterial contamination	22
Stage of lactation	23
Mastitis	23
Application of the Phosphatase Test to Commercial Holder Pasteurization	23
Application of the Phosphatase Test to H.T.S.T. Pasteurization	26
Application of the Phosphatase Test to Products other than Milk	30
Cream	30
Skim milk, etc.	32
Butter and butter milk	33
Cheese	34
Ice Cream	35
Summary and Conclusion	37
Appendix.—Methods for performing the Phosphatase Test	39
Precautions	39
A. Technique of the Kay-Graham test	39
B. Neave's modification	42
C. Gilcreas and Davis' modification	42
D. Scharer's modification	44
E. Leahy's modification	48
F. Aschaffenburg and Neave's modification	49
References	52

F 8,30:6;8;a17

CFTRI-MYSORE

503
Phosphatase test

N39

INTRODUCTION.

Heating of milk has been the main commercial method used in many countries during the last few decades to improve its keeping quality and to ensure that it can be handled and distributed on a large scale with the minimum of losses from souring. The temperatures used have varied widely in different countries—between 55(131)°* and 85(185)°—and the time of treatment has also varied.

Further, *heating done under appropriate conditions* is undoubtedly effective in destroying pathogenic micro-organisms which may be present in the raw fluid. This has become of increasing importance during the last twenty years and, at least in the public mind, now outweighs the importance of the original commercial necessity for heating, namely the extension of the keeping life of raw milk. The keeping life of raw milk is in any case far greater today than twenty years ago because methods of farm production and of farm and depot cooling have improved and the need for bacterial cleanliness in all vessels with which milk comes into contact, both on the farm and during collecting and distribution, has been realized. Consequently the heating of milk, from the purely commercial standpoint, has become relatively less important.

Pasteurization of milk may for present purposes be defined as heating under time-temperature conditions which will ensure the complete destruction of pathogenic micro-organisms of any type likely to be present in raw milk. It may be almost useless as a safeguard to the consumer against possible infection unless these time-temperature conditions are rigidly controlled. Two principal methods of control are available, one the close supervision of process and plant, the other the examination of the pasteurized product. Absolute safety in pasteurization may be, like most other absolutes, unattainable; it is certain that even the strictest supervision and mechanical control (automatic temperature control, recorders, etc.) cannot by themselves ensure freedom of the pasteurized product from serious faults. Increasing attention has therefore recently been directed to the second of the two methods of control, *viz.* the detection of even minor faults by examination of the pasteurized milk.

Milk is well known to undergo a number of chemical, physico-chemical and enzyme changes under the influence of heat, and many methods can be and have been worked out to demonstrate such changes (see the recent survey of Scharer (1)).

Most methods which distinguish between raw and heated milk fail when met with the more exacting demand of showing up *minor* faults in pasteurization or of demonstrating the presence of *small* quantities of raw milk in the pasteurized product. Since these minor defects in pasteurization may be almost as great a potential source of danger to the consumer as are gross errors, no method of control which fails to indicate them can be regarded as sound. The task of finding a satisfactory method of control is complicated by the fact that time-temperature regulations for pasteurization vary in different countries. A method, to be successful, should be adaptable to these variable conditions. In particular it will be of advantage if the method can be applied equally to products pasteurized by the low temperature—long time (" holder ") or the high temperature—short time process, since these processes are frequently practised side by side.

Critical examination has shown that none of the proposed methods of control based on physico-chemical changes can be said even to approach fulfilment of these

* Temperatures throughout are given in centigrade and, bracketed, in Fahrenheit degrees.

requirements. (Orla-Jensen's promising "creaming test" (2), for example, is unfortunately not applicable to homogenized milk nor to milk from those breeds of cows, such as the Jersey, which produce large fat globules.)

More promise is shown by the type of test based on destruction of enzymes. Here again critical study has greatly reduced the number of possibilities. The tests may be divided into those based on *partial* destruction of an enzyme and those based on *complete* or practically complete inactivation of an enzyme. Tests in which the residual amount of enzyme after heating is used to indicate efficiency of pasteurization are severely handicapped by the fact that the amount of any enzyme in raw milk usually varies considerably. The catalase, the aldehyde-reductase and the amylase tests are examples of this type.

Catalase is very stable to heat, complete inactivation occurring only after heating to 90(194)–92(197·6)° for 20–30 minutes (3). Methods for controlling efficiency of pasteurization have been proposed, based on the (gas volumetric) determination of residual catalase (4). Since the catalase content of raw milk varies considerably (4) and partial re-activation is liable to take place on standing (3, 4), the catalase test is not accurate enough for the clear cut distinctions required in the efficient control of pasteurization.

Aldehyde-reductase (Schardinger's enzyme) is only slightly less stable to heat than catalase. Complete inactivation occurs after heating to 75(167)° for 20 minutes (5). Partial destruction of this enzyme, as shown by the time required to reduce formalin-methylene blue solutions, was the basis of a proposed test (6). Bengen and Bohm (7), after investigating the test under carefully controlled conditions, came to the conclusion that it is of little value in the control of pasteurization, since the amount of enzyme in raw milk is far from constant. They found, moreover, that it is not possible to detect by this test the addition of even a large percentage of raw milk to pasteurized milk.

Amylase (diastase) is less stable to heat, 78 per cent. of the enzyme being destroyed by heating milk to 63 (145·4)° for 30 minutes (8). The hydrolytic activity of this enzyme on soluble starch, as indicated by a starch-iodide reaction, formed the basis of tests proposed by Rothenfusser (9), Gould (10) and others. Kluge (8) found that in milk heated to 55(131)° for 30 minutes the reduction in enzyme activity was almost equal to the reduction at 60(140)–63(145·4)°. This was confirmed by Richardson and Hankinson (11). Underpasteurization cannot therefore be detected with any degree of certainty. Other faults of the tests based on this enzyme were demonstrated by Gilcreas and Davis (12), and it may be concluded that the amylase test is unsuitable for effective control of pasteurization.

Total destruction of the pathogenic micro-organisms which may be present in raw milk is the chief aim of legal pasteurization and controls the choice of conditions for effective pasteurization. The value of tests depending on *complete* inactivation of an enzyme will clearly be associated with the relationship between the thermostabilities of the enzyme and of the most resistant pathogenic organisms. A test founded on complete destruction of an enzyme less stable than the organisms will be less useful than one founded on the destruction of an enzyme slightly *more* stable than the most resistant of the pathogens (*Myco. tuberculosis*). In the latter case inactivation of the enzyme will not be complete until the time-temperature requirements for destruction of all the pathogens have been exceeded. Methods based on complete inactivation will obviously be more sensitive to the addition of raw milk to pasteurized milk than methods based on partial inactivation.

In a restricted way peroxydase can be regarded as an example.

Peroxydase : the tests proposed by Storch (13) and Rothenfusser (9) and various modifications of these tests are based on the observation that on the complete destruction of the enzyme a colour reaction, given when even small amounts of peroxydase are present, disappears. As, according to Bouma and van Dam (14), peroxydase is completely destroyed only when milk is heated to 70(158)° for 150 minutes, or 75(167)° for 2·5 minutes, or 80(176)° for 2·5 seconds, these methods are not applicable to " holder " pasteurized milk (heated at 62·8(145)° for 30 minutes). Moreover, since high temperature—short time pasteurization is nowadays frequently performed at a temperature as low as 71·1(160)° with a holding time of only 12–20 seconds, the applicability of the test becomes further limited to cases in which heating to temperatures above 80(176)° is still required.

No other test belonging to the second type was known until in 1933 Kay and Graham (15) showed that another enzyme, *phosphatase*—for which straightforward and fairly sensitive methods of detection and measurement already existed—forms a regular constituent of raw milk and that its thermostability conforms closely to the requirements outlined above. Careful study has led to modifications and improvements of the relatively crude test proposed in 1933, so that the modern phosphatase test provides a far better method of controlling pasteurization than any of those previously mentioned.

PHOSPHATASE.

According to Folley and Kay (16) phosphatases may be defined as those enzymes which catalyse the scission or formation of linkages (usually ester linkages) involving completely oxidized phosphoric acids (ortho-, meta-, or pyro-). Amongst substrates for their activity are esters such as glycerophosphates, hexosephosphates, nucleotides, and phenyl phosphate. Phosphatases are widely distributed amongst animals, plants and micro-organisms and their presence in a great number of mammalian organs, tissues and secretions has been established. For detailed information the reader is referred to the recent review by Folley and Kay (17).

Differences in the hydrolytic action on various substrates and in the optimum pH for hydrolysis have led to the recognition of several types of phosphatase, of which the " phosphomonoesterase A_1", with an optimum activity at a pH of 9–10, may be said to be one of the most widely distributed.

Milk Phosphatase.

The presence of a phosphomonoesterase of type A_1 in cow's milk was clearly established by the work of Graham and Kay (18), after previous observations by Demuth (19) and by Wilson and Hart (20). Further investigations on milks from a great number of cows of different breeds, ranging over the whole lactation period, proved conclusively that phosphatase is invariably present in raw milk (21). It was found that the concentration of phosphatase in milk changes in a regular manner during the progress of lactation : after falling to a minimum during the first 15–25 days *post partum* the concentration increases steadily throughout the rest of the lactation period. The phosphatase concentration—lactation period curve is roughly the inverse of the milk yield—lactation period curve.

The facts that the concentration of phosphatase in milk is higher than its concentration in blood serum or plasma (20, 21) and that no correlation exists between daily or periodical changes in plasma phosphatase and in milk phosphatase (21, 22) make it unlikely that the presence of phosphatase in milk is due simply to diffusion from the blood. It seems more probable that the milk phosphatase originates from the secreting cells of the mammary glands which form a rich source of the enzyme (23, 24).

From consideration of the rate of secretion of phosphatase in milk at different stages in the lactation cycle, supported by evidence derived from a study of the effect of thyroxine on lactating cows, Folley and Kay (21) concluded that, in general, the amount of phosphatase secreted in unit volume of milk may be regarded as an inverse index of the efficiency of the mammary gland, the more efficient cell losing less of its enzyme furniture for the secretion of a given volume of milk than the less efficient.

So far no specific function of the phosphatase *in milk itself* has been discovered. That such a function exists seems unlikely when it is remembered that milk phosphatase shows an optimum activity at a pH in the neighbourhood of 9, *i.e.* at a much higher pH than that of milk (pH 6·5–6·7). Nevertheless, the phosphoric esters in milk are hydrolysed to a considerable extent (30–50 per cent.) when milk is kept at body temperature for four hours (18), an observation which makes it conceivable that significant changes may still take place in these esters while the milk is accumulating in the ducts and cisterns of the udder after leaving the secretory cells of the mammary gland. Any autolysis outside the udder may be suppressed by keeping the milk at low temperatures.

Kay and Graham (15) found that whilst in milk the phosphatase tends to concentrate in the fatty layer, it is in the aqueous layer that the enzyme becomes highly concentrated when molten butter is centrifuged. Their view is that the enzyme is largely associated with the surface of the milk fat particles, being adsorbed by or incorporated in the protein layer surrounding the fat globules. When the state of emulsion of the fat changes, as during the production of butter, the phosphatase leaves the fat altogether. Its high concentration in the aqueous phase of butter speaks against the solubility of the enzyme in the fat itself.

The thermal and other stabilities of the phosphatase *in milk* are clearly of first importance when considering the possible utilization of the enzyme as an index of pasteurization. The enzyme appears to be quite stable for days or even weeks when milk is kept under storage conditions (1), but is destroyed with increasing rapidity as the temperature of the milk rises above body heat. Destruction is slow below 60(140)° but the time required for complete destruction falls from more than 1 hour to a second or two over the critical range from 60(140)° to 75(167)° at which pasteurization by either the low temperature—long time or the high temperature—short time process is generally practised, whilst the enzyme is almost instantaneously inactivated at temperatures above 75(167)° (15). The rapidity with which the rate of destruction increases as the temperature is being raised is well demonstrated in Figure I, obtained from an investigation by Koppejan (25) who showed that for the temperature range 58(136·4)–62(143·6)° the enzyme destruction follows the course of a mono-molecular reaction, according to the equation

$$C_t = \frac{C_o}{e^{at}}$$

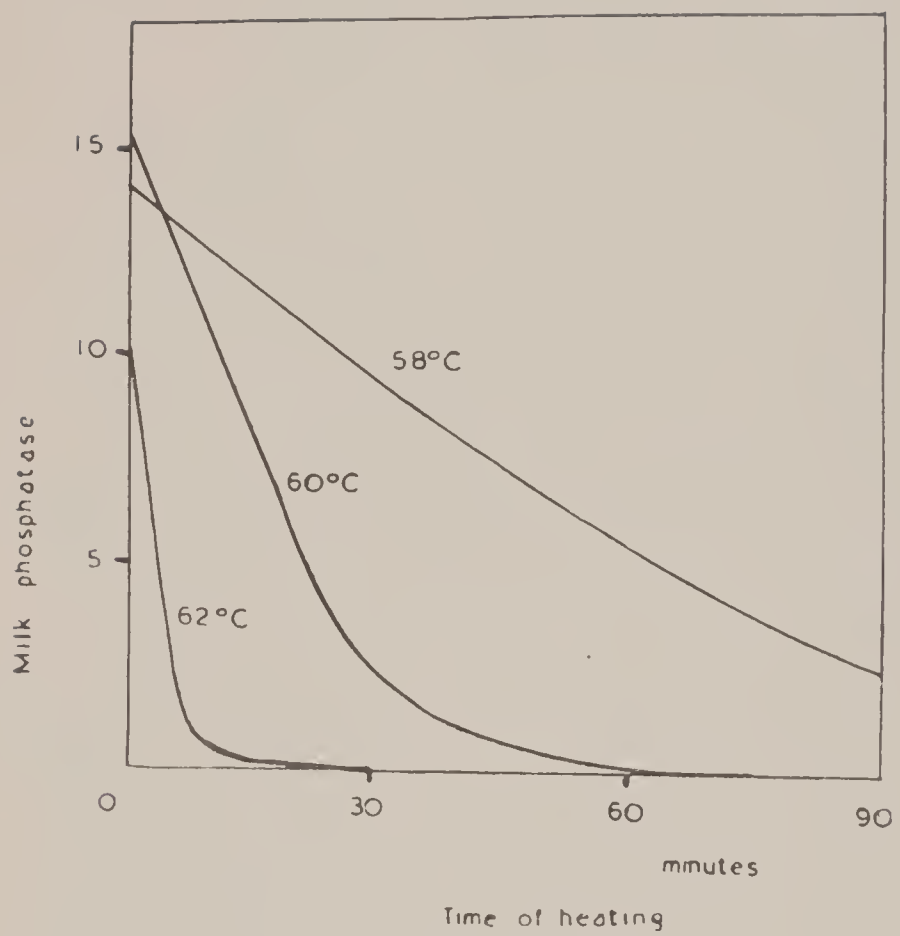
where

C_o = initial phosphatase activity
C_t = phosphatase activity after time t
t = time in minutes
a = reaction constant

The reaction constant, a, was found to be approximately doubled by an increase in temperature of 1(1·8)°.

It has been pointed out already that the main requirement for a pasteurization test based on practically complete destruction of an enzyme is that the heat resistance of the enzyme should be slightly, but only slightly greater than the thermostability of the pathogenic organisms likely to occur in milk. That this requirement is fulfilled

FIGURE I.



by milk phosphatase was shown by Kay and Graham (15) who, taking *Myco. tuberculosis* as the most heat resistant of these pathogens, compared its death rate with the rate of destruction of phosphatase with the result shown in Figure II.

The figure shows that over the whole temperature range from 60(140)° to 75(167)° total destruction of the tubercle bacillus occurs *before* 96 per cent. destruction of the phosphatase. Absence of phosphatase from a sample of pasteurized milk will therefore be indicative of complete extermination of the pathogenic micro-organisms which may have been present in the original raw milk.*

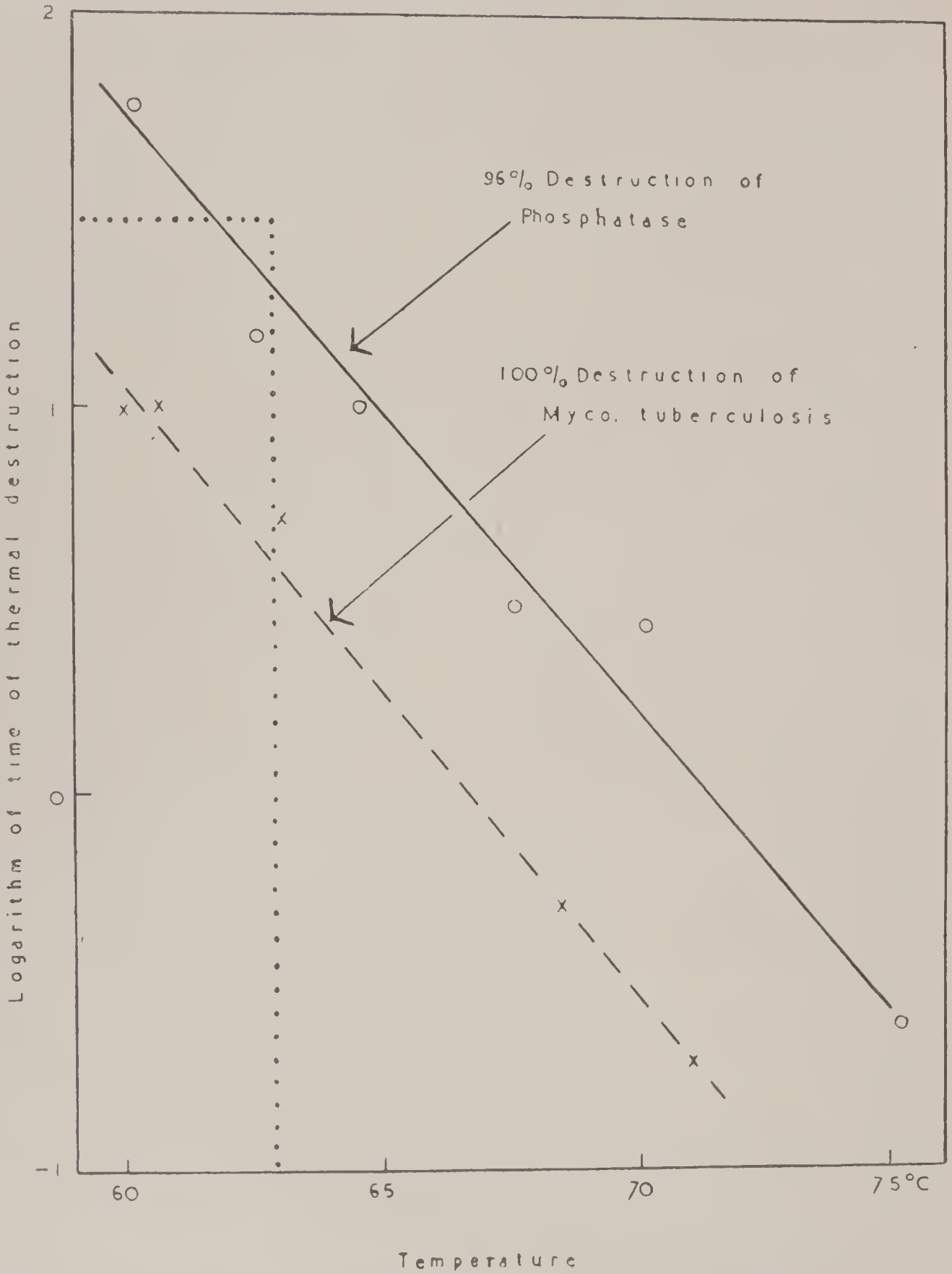
PHOSPHATASE DETERMINATION IN MILK.

General technique for phosphatase determination.

Methods for the estimation of phosphomonoesterase activity have been developed over a number of years, a development stimulated in part by the discovery of the relationship between the amount of phosphatase in unit volume of blood plasma and the extent of pathological changes in certain diseases, particularly in bone disease. The enzyme was found to be of assistance in the diagnosis and control of such diseases.

* Unless minute quantities of raw milk, insufficient to give a phosphatase reaction but containing pathogenic micro-organisms, have found their way into the milk after pasteurization, as is conceivable.

FIGURE II.



The dotted vertical and horizontal lines indicate the minimum temperature and time for official pasteurization in Great Britain.

The majority of the methods for phosphatase determination relied on the same principle: addition of the material to be tested to a standardized substrate (monoester of phosphoric acid) either with or without addition of a buffer solution, incubation (usually at 37(98·6)°) for a definite period and subsequent determination of the liberated inorganic phosphate by gravimetric or colorimetric methods.*

Original method.

Following their observation that milk contains an "alkaline" phosphatase which seemed to be destroyed by pasteurization (18), Kay and Graham (15), in 1933, adapted the above principle to milk. They worked out a qualitative and a quantitative method for the estimation of phosphatase in milk, using Na-β-glycerophosphate in a glycine buffer as substrate, and determining the inorganic phosphate colorimetrically by means of Briggs' (26) reagent. This test, however, was not very sensitive to slight defects in pasteurization. Its limitations were soon realised and, in 1935, a drastically changed method was introduced by the same authors (27).

The original method is still in occasional use in the modified form given by Stein (28), but, although certain improvements were made, Stein's modified and improved technique lacks the high degree of sensitivity attained by the 1935 method (27) just mentioned, and by other methods (29).

The "Kay-Graham" test.

Shortly after the publication of the original phosphatase test, King and Armstrong (30) showed that the substrate disodium phenyl phosphate is hydrolysed by the phosphatase of blood plasma at a quicker rate than Na-β-glycerophosphate and that greater sensitivity and convenience could be attained by determining the liberated free phenol (with Folin's reagent (31)) rather than the inorganic phosphate. These innovations in the blood plasma technique were incorporated in the revised method for milk (which will be referred to as the Kay-Graham test). In addition the glycine buffer was replaced by Na-diethyl barbiturate ("Na-veronal") (32) and the time of incubation was altered. As a result two tests emerged, one a simple qualitative one, completed in half an hour, which allowed the detection of any gross errors in pasteurization methods, and the other a precise quantitative test capable of indicating quite minor faults in pasteurization. A description of the methods in their up-to-date form will be found in Appendix A (see also Appendix B).

Kay and Graham found that on testing milk properly pasteurized for 30 minutes at 62·8(145)° a blue colour was developed which varied amongst different samples between 1·3 and 2·2 blue units when measured under standard conditions with the Lovibond tintometer. They decided on 2·3 Lovibond blue units as being a reasonable limiting value for correctly pasteurized milk, and subsequent experience has amply justified this choice.

As the blue colour in corresponding control tests generally amounts to 1·0–1·5 blue units only, traces of residual phosphatase remain active under the conditions of the test.

Since the Kay-Graham test gives values of the order of 50 blue units when carried out on raw milk, it might appear as if one per cent. or more of the original phosphatase remained active in properly pasteurized milk. This is, however, not the case. As will be shown later, the addition of only 0·5 per cent. of raw milk to a properly pasteurized sample raises the blue value from below 2·3 to 4–6 Lovibond units, showing that the enzyme concentration—phenol concentration curve rises very steeply over the range of small enzyme concentrations. The colour difference of about 0·5 Lovibond units

* For a summary of these methods see Folley and Kay (17).

between control tests and tests on properly pasteurized milk corresponds in reality, therefore, to a quite minute amount of residual phosphatase. That traces should remain active is to be expected in view of the mechanism of the phosphatase destruction. As already mentioned, the inactivation follows the course of a monomolecular reaction and from a consideration of the equation given on page 6 it becomes clear that, in theory, complete inactivation ($C_t=0$) will only occur after a time $t=\infty$.

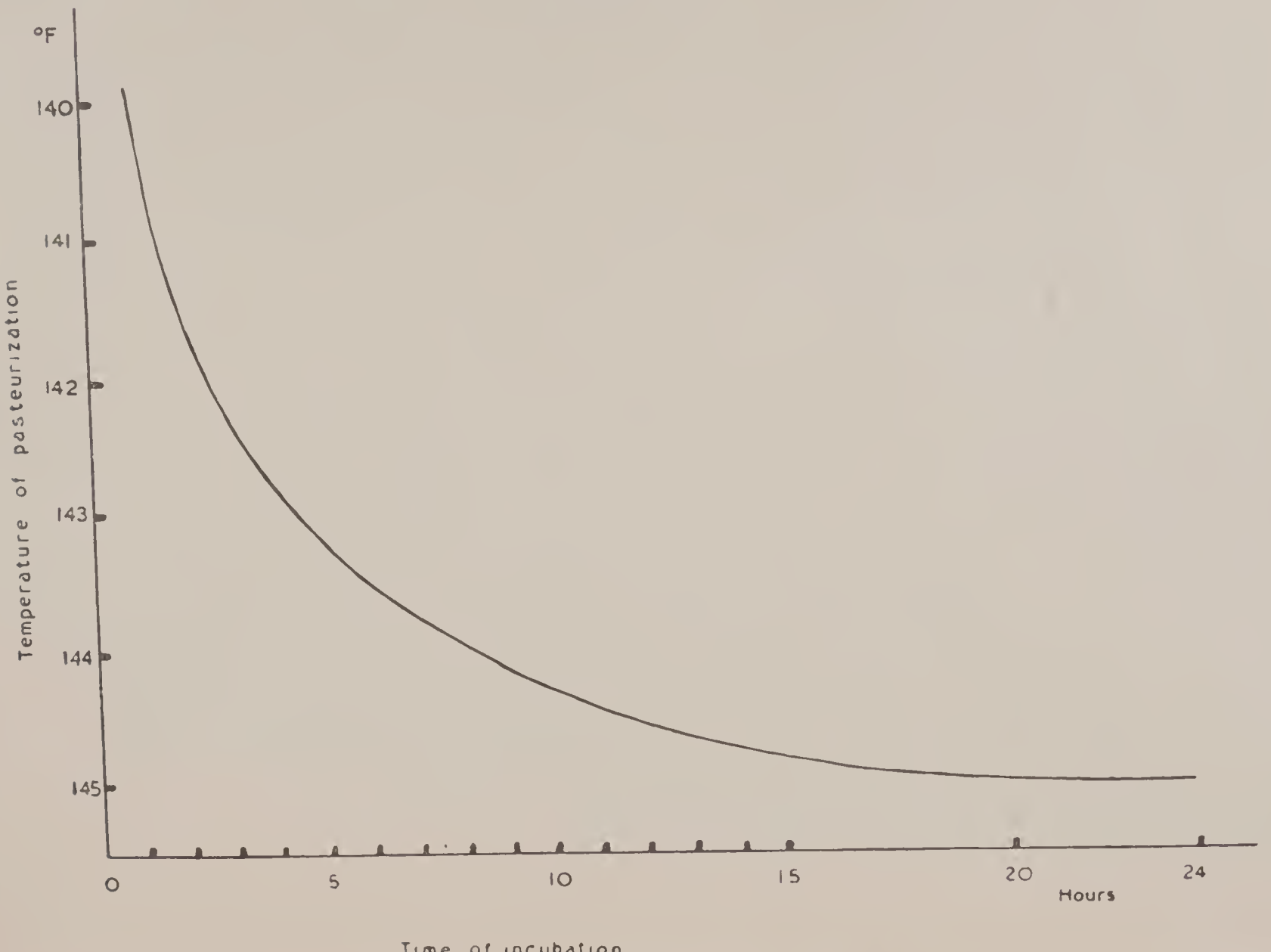
It follows that the lower the temperature of pasteurization, the more one tends to lose the advantages derived from complete destruction of the enzyme which have been outlined already (see page 4). It is, therefore, from the point of view of control by the phosphatase test, unfortunate that in other countries (for instance Canada and the United States) lower minimum temperatures down to 60(140)° have been adopted for pasteurization. It has been possible, however, to overcome the difficulties thus presented. The adaptation of the Kay-Graham test to temperatures lower than 62·8(145)° has been accomplished in two different ways, either (*a*) by shortening the time of incubation whilst retaining the original colour standard of 2·3 Lovibond blue units, a method proposed by Kay and Graham (27), or (*b*) by retaining the original incubation time of 24 hours whilst choosing a higher colour standard, a method introduced by Gilcreas and Davis (12).

As regards (*a*), Kay and Graham recommended adjustment of the time of incubation in accordance with Figure III.

Their proposal has been more or less closely followed by a number of investigators. Thus Krueger (33, 34) reports the satisfactory working of the Kay-Graham test when using an incubation time of 8 hours for milk pasteurized at 62·2(144)° for 30 minutes, as required in Chicago. Good results were obtained by Smith (35) with an incubation time of $4\frac{1}{4}$ hours for milk pasteurized at a minimum temperature of 61·7(143)°. Working under the same conditions, Geiger and Davis (36) reduced the period of incubation to one hour. No explanation is given for this drastic reduction which, of necessity, deprives the test of some of its sensitivity. Spencer (37) tested milk pasteurized at a minimum temperature of 61·1(142)° without being "able to detect any appreciable difference between an incubation time of six hours and one of 24 hours". This points to a serious fault in technique, as Storrs and Burgwald (38) who made an extensive study of the Kay-Graham test, showed in a series of experiments with milk pasteurized at 61·1(142)° that the amount of blue colour developed in the phosphatase test *does* increase considerably when the period of incubation is lengthened. Thus they found an average increase in blue colour of 22·7 per cent. when using an incubation time of 5 instead of $2\frac{1}{2}$ hours and an average increase of as much as 86·5 per cent. when incubating for 24 instead of for 5 hours.

Storrs and Burgwald heated milk at various temperatures between 60(140)° and 62·8(145)° for periods varying from 20 to 40 minutes and determined the amount of blue colour developed after incubation for $2\frac{1}{2}$, 5 and 24 hours. Their findings are remarkable in the surprisingly low colour readings which are reported. Storrs and Burgwald found, for instance, that milk heated for only 20 minutes at 62·8(145)° gave in the majority of cases blue values below 2·0 Lovibond units after incubation for 24 hours and that milk heated for 30 minutes at 61·7(143)° gave, on the whole, values below 2·3 Lovibond units after the same period of incubation. (See their Tables VI and VII (the headings of which have been reversed, due to a printer's error)). These figures would indicate that it is not possible to detect a shortening of heating time of 10 minutes or a drop in the pasteurizing temperature of 1·1(2)° when dealing with milk required to be pasteurized at 62·8(145)°, whereas there is no doubt that such inefficiencies are, in general, detectable by the Kay-Graham test. In a more recent paper Burgwald and Gilbertson (39) applied the Kay-Graham test to milk pasteurized at 61·7 (143)°, using an incubation time of 24 hours instead of the $4\frac{1}{2}$ hours recommended by Kay and Graham, and the figures quoted are again inexplicably low.

FIGURE III.


°F
140
141
142
143
144
145
Temperature of pasteurization
0
5
10
15
20
24
Hours
Time of incubation.

Some anomaly in the technique used by Burgwald and his collaborators must, therefore, be suspected.

One drawback to Kay and Graham's proposal to reduce the time of incubation when applying the phosphatase test to milk pasteurized at temperatures *below* 62·8(145)° is the reduced sensitivity to the presence of raw milk which follows. Since the hydrolysis caused by raw milk will be independent of the temperature at which the pasteurized milk has been previously held, the blue colour produced by small additions of raw milk will be far less pronounced if samples are, for instance, incubated for only $2\frac{1}{2}$ instead of 24 hours.

Gilcreas and Davis' modification.

Whilst a number of investigators followed Kay and Graham's proposal of adjusting the time of incubation to the temperature of pasteurization, Gilcreas and Davis (12) preferred the alternative method of retaining the 24 hours' incubation time (which has certain advantages from the standpoint of organisation within the laboratory) and of raising the limit of blue colour permissible in properly pasteurized milk. (Their technique is described in appendix C). Instead of Lovibond blue units they used permanent colour standards prepared from inorganic salt solutions and expressed the colour readings in mg. of phenol per 0·5 ml. of milk. The limiting value for milk pasteurized at 61·7(143)° for 30 minutes was, at first, fixed at 0·037 mg. of phenol, this being the average figure obtained from 20 experiments which gave values between 0·03 and 0·05 mg. This limit seemed to be rather low, and, as Smith (35) has pointed out, " it would appear fairer to the operators of pasteurizing plants, if the practice of Kay and Graham was followed and the maximum figure rather than the average chosen as standard". In a collective experiment, reported by Gilcreas (40) the higher limit of 0·05 mg. was, in fact, employed to good purpose, but the " tentative method " of the American Association of Official Agricultural Chemists (41) which is based on Gilcreas and Davis' modification has reverted again to a lower limit of 0·04 mg. As a result of further experience with the test, Gilcreas (42) now recommends the adoption of a standard of 0·05 mg. of phenol per 0·5 ml. of milk.

No accurate figures are available showing the relationship between the phenol standard and the Lovibond blue unit. Storrs and Burgwald (38) state that " Gilcreas and Davis concluded that the production of a phenol value equal to 0·037 mg. of phenol per 0·5 ml. of milk is equivalent to 2·3 standard Lovibond blue units", but no such conclusion is contained in the reference cited (12) and it is unreasonable to assume that milk pasteurized at 61·7(143)° instead of 62·8(145)° should give average colour readings of 2·3 blue units, *viz.* equal to the limiting amount for milk pasteurized at 62·8(145)°. From graphs given by Anderson, Herschdörfer and Neave (43) it appears that 0·037 mg. of phenol per 0·5 ml. of milk correspond to approximately 3 Lovibond units whilst 0·05 mg. of phenol per 0·5 ml. of milk are equivalent to about 4 blue units under the conditions of the test. In mixed milks pasteurized at 61·3(142·5)°, *viz.* at a temperature which is only slightly lower than that studied by Gilcreas and Davis, Kay and Graham (27) found values of 4·4–5·0 Lovibond blue units, so that the higher limiting value of 0·05 mg. of phenol per 0·5 ml. of milk, as now recommended by Gilcreas (42), seems appropriate. It was said that no accurate figures are available showing the relationship between Gilcreas and Davis' colour standards and the Lovibond blue unit. This statement must be upheld in spite of the fact that Burgwald and Gilbertson (39) have provided extensive sets of figures comparing Gilcreas and Davis' modification with the Kay-Graham test. As has been mentioned before (see page 12), these authors obtained inexplicably low colour readings with the latter test although values of the right order were given by them for the colour readings obtained with the Gilcreas and Davis technique.

Apart from its adaptation to lower temperatures of pasteurization, the actual technique of the Kay-Graham test has, until recently, been little altered since its introduction. Its convenience has been enhanced by the fact that the necessary reagents are now available from commercial sources, buffer and substrate being combined in the right proportions in tablet form. According to Kay and Neave (44) Whatman filter paper No. 42 is preferable to No. 30 but has been found slow in filtration (45) so that, as in America (41), the use of No. 40 is now recommended.

Research has been going on for some considerable time at the National Institute for Research in Dairying, Shinfield, near Reading, England, with the aim of simplifying the technique of the Kay-Graham test and as a result a modification has been developed which makes it possible to dispense with the (second) filtration process after the addition of Na_2CO_3 (45). This is achieved by adding sodium hexametaphosphate to the phenol reagent. Its presence prevents the formation of the precipitate normally formed on boiling after the addition of Na_2CO_3. Time and material are thus saved and results are obtained which agree closely with those of the original test. The necessary alterations in the technique of the Kay-Graham test are given in Appendix B.

Methods of colour reading.

Whilst the actual technique has thus remained practically unchanged, a number of proposals have been put forward regarding the method of evaluating the amount of blue colour produced in the test. In the original paper Kay and Graham suggested two alternatives where a tintometer was not available : either comparison in a 13 mm. cell against a standard glass disc made to correspond to 2·3 Lovibond blue units, a method which, of course, only allows distinction between properly and improperly pasteurized samples, or comparison by means of a colorimeter, a method necessitating the preparation of accurate phenol standards. As standards prepared from phenol and Folin's reagent are not stable, the additional labour of preparing such standards at frequent intervals makes the use of the colorimeter less attractive. Anderson *et al.* (43) showed that more permanent standards may be obtained by matching phenol standards with colour solutions made up from organic (chlorazol) dyestuffs.

Herschdörfer and Neave (46) constructed a simple comparator in which the blue colour developed in the test samples is compared in 25 mm. depths with either the colour of a single glass disc corresponding to 2·3 Lovibond units or the colour of three discs corresponding to 1·5, 2·3 and 6·0 Lovibond units. The latter type allows the checking of control tests against the 1·5 disc as well as the classification of the actual samples tested into 3 groups :—

(i) properly pasteurized	=	$<$2·3 units
(ii) improperly pasteurized	=	2·3–6·0 units
(iii) gross errors in pasteurization	=	$>$6·0 units

This device has been found very useful in the practical control of pasteurization in Great Britain.

Burgwald and Gilbertson (39) used a photoelectric cell for the determination of the blue colour developed in the Kay-Graham test. The device, which requires careful standardization, was found to have no particular advantage apart from the elimination of the human element.

The inorganic colour standards, introduced by Gilcreas and Davis, have already been mentioned (see page 12). They were originally devised for use in daylight only, but it was later shown that accurate readings may be obtained in artificial light also, if a daylight lamp is used and its light reflected from a blue glass plate (47). A comparator incorporating this principle has been constructed by Boynton and Nelbach (48). For accurate measurements it is necessary to match the colour in test tubes of a uniform diameter of 13 mm. It is, therefore, important to collect the filtrate *after*

the addition of the Na_2CO_3 solution in test tubes of this diameter and *not* before, as stated in the " tentative method " of the American Association of Official Agricultural Chemists (41).

Scharer's modification.

An important modification of the Kay-Graham test in which the actual technique is involved was introduced by Scharer (1). The principle is, of course, the same and the substrate, disodium-phenylphosphate, is retained, but a borate buffer replaces the " Na-veronal", the milk proteins are precipitated with basic lead acetate, and Gibbs' phenol reagent, 2 : 6-dibromoquinone chloroimide (49), is preferred to Folin's reagent on account of its greater specificity and sensitivity. The sensitivity is given as one part in 20 millions, but it should be pointed out that this figure relates to spectrophotometric and not to colorimetric methods. As in the original Kay-Graham test, two methods are provided, an accurate laboratory test and a qualitative (field) test. Both methods have been improved and simplified since they were first introduced (50, 51, 52). Their most recent form is given in Appendix D.

In the quantitative method, the time of incubation is reduced to 1 hour when dealing with milk pasteurized for 30 minutes at 61·7(143)°, and only one filtration process is required. Adequate pasteurization is indicated by the absence of any appreciable amount of blue colour and in consequence greater purity of the reagents, especially of the substrate, becomes a necessity.

Special precautions had, at first, to be taken to ensure the absence of any colour-producing substances, but it appears (51) that substrate preparations are now commercially available which are sufficiently pure to obviate such precautions. Difficulties caused by the instability of the reagents have, at least in part, also been overcome. Reagents in tablet form are now obtainable.

In order to estimate the degree of underpasteurization, the colour developed in test samples was originally compared with a set of standards made up from phenol solution and Gibbs' reagent which, contrary to those prepared with Folin's reagent, were reported to be stable for weeks, if kept cool and protected from the influence of direct sunlight. These were later replaced by more stable colour standards made up from inorganic salt solutions (51). Alternatively a single standard corresponding to the limiting amount of blue colour permissible for milk correctly pasteurized at 61·7(143)° may be made up weekly from a solution of sodium sulfocarbolate (51). Recently still another set of standards covering a far greater range of blue colour has been developed (81). These standards are prepared from solutions of inorganic salts in ammonium hydroxide.

The amount of colour produced by 0·001 mg. of phenol in 5 ml. of solution is chosen as the unit of blue.

For the reading of the indophenol colour, Tracy and Hahn (53, 54) applied the photoelectric cell with good results.

The results (1, 39, 53, 54) obtained when applying Scharer's laboratory test to milk required to be pasteurized at 61·7(143)° are comparable with those obtained by the Kay-Graham test and the test appears to work equally well when used under different conditions, for instance, in the testing of milk pasteurized at a minimum temperature of 63(145·4)°, as was recently shown by Jepsen and Madelung (29). These authors compared Scharer's modification with the Kay-Graham test and found the former superior in sensitivity. In order to achieve this result it was, however, necessary to raise the limiting colour standard and to increase the period of incubation from 1 hour to 20 hours, so that the main advantage claimed for Scharer's method, *viz.* the rapidity with which the test can be completed, was lost. Using the limiting colour standard recommended by Scharer and an incubation period of 3 hours, Jepsen and Madelung found Scharer's method less sensitive than the Kay-Graham test.

Aschaffenburg and Neave (55) who studied the possibilities of adapting Scharer's method to pasteurizing conditions in Great Britain obtained results indicating that an incubation period of about 3 hours would probably be adequate. They abandoned this adaptation in favour of a more rapid method which is dealt with below.

As already mentioned Scharer also developed a *short* test in which the technique is still further simplified (1). In this modification the incubation time has been reduced to as little as 10 minutes and the need for any filtration has been avoided. The test has been subject to considerable improvements since it was first introduced (50, 51), particularly with regard to the method of colour reading. Whereas originally the unfiltered samples were directly examined for the presence of any blue colour indicative of underpasteurization, the indophenol colour is now extracted into butyl alcohol and compared with two colour standards, made up from inorganic salt solutions, of a strength corresponding to slight faults in pasteurization. In this way the sensitivity has been greatly improved, since the colour shows up much better in the clear alcoholic layer. No blue or blue-green colour should develop in samples properly pasteurized at 61·7(143)°. Magnesium, which has been known for some time to catalyse the hydrolysis by phosphatase (see (17)), was at one time utilised in the test (50), but is now no longer employed as its presence gave rise to difficulties.* Purity and stability of the necessary reagents have been improved and, according to Scharer (50), the test is now comparable in accuracy either to his laboratory modification or to the Kay-Graham test. Burgwald and Gilbertson (39) found the test slightly less effective than the Kay-Graham test. Roger (56), who applied the test under commercial conditions with satisfactory results, recommends the use of amyl alcohol instead of butyl alcohol for the extraction of the indophenol colour, as less yellow colour is taken up by this solvent from highly coloured milk. It is necessary to prepare special colour standards when amyl alcohol is used, since less colour is extracted by this solvent. Roger found it possible to increase the sensitivity of the test by lengthening the time of incubation to 30 minutes. Experiments showed that this causes a marked increase in the amount of colour developed in underpasteurized samples whilst having little effect on properly pasteurized samples. The more distinct colour differences were easier to distinguish in artificial light. Incubation for only 10 minutes is, however, undoubtedly a great advantage when it is intended to use the test for its original purpose, *viz.* as a field test.

Other modifications.

Leahy (57, 58) obtained good results with a modified test which is best described as a combination of Scharer's modification and the Kay-Graham test. Milk is incubated for 18 hours with the buffer substrate used in the Kay-Graham test, but the colour is then developed with Gibbs' phenol reagent and read directly without further filtration. The technique is described in Appendix E.

Since with Scharer's short test no colour is developed in milk heated for 30 minutes at 61·7(143)° and since it is necessary to recognise such milk as underpasteurized when dealing with samples pasteurized in accordance with the somewhat higher requirements valid in Great Britain, alterations in Scharer's technique are required in order to detect the smaller quantities of enzyme remaining active after heat treatment at temperatures *above* 61·7(143)°. Aschaffenburg and Neave (55) found that this necessitated an increase in the period of incubation to 1 hour at 37(98·6)° or to 40 minutes at 47(116·6)°. As a field test of this duration is hardly practicable, they have incorporated certain alterations in reagents and technique which make for higher accuracy and have recently developed a laboratory modification for the testing of milk

* Private communication by H. Scharer.

required to be pasteurized for 30 minutes at a minimum temperature of 62·8(145)°, which requires an incubation period of only 1 hour and has been found to be as searching as the Kay-Graham test. The method is described in Appendix F.

SENSITIVITY OF THE PHOSPHATASE TEST.

In order to evaluate the sensitivity of the phosphatase test, carefully controlled laboratory experiments are required to determine the minimum deviations from the official pasteurizing conditions which can be detected by the test, i.e. which will lead to the development of blue colour in excess of the amount permissible in milk known to be efficiently pasteurized. Tests carried out on commercially pasteurized samples will not, in general, serve this purpose.

In commercial pasteurization the three most common faults are (i) heating of milk to temperatures below the required minimum or (ii) heating for a shorter period than demanded by regulation or (iii) presence of raw or only partially heated milk in the pasteurized product. Heating to too high a temperature or for too long a time are infrequent faults, since they tend to damage the cream line. They are, in any case, of little public health significance. Laboratory experiments have been conducted to examine the sensitivity of the phosphatase test to each of the variables (i), (ii), (iii), mentioned above. The results will be dealt with in this order.

(i) *Sensitivity with regard to temperature of pasteurization.*

This is determined by heating milk samples for the full pasteurization time of 30 minutes to temperatures lower than the minimum required for efficient treatment.

Kay and Graham (27) obtained the results given in Table I by heating a number of milks for exactly 30 minutes at different temperatures.

TABLE I.

Effect of temperature of pasteurization on development of blue colour in the Kay-Graham test ("B"). Milks pasteurized at temperatures from 58·6(137·5)° *to* 62·8(145)° *for exactly* ½ *hour.*

Serial number of milk sample	Temperature of pasteurization			
	58·6(137·5)°	60(140)°	61·4(142·5)°	62·8(145)°
	Lovibond blue units			
41*	35·0	19·4	4·4	1·8
42*	33·5	22·8	5·0	2·0
43*	31·0	22·5	4·5	1·8
44*	32·5	21·0	4·6	2·1
45	9·6	3·7	2·3†	1·9
46	30·5	12·2	3·1	1·6
47	28·5	23·7	3·1	1·7
48	12·8	6·4	2·2†	1·8
49	30·6	24·0	3·0	1·8
50	25·2	23·4	4·2	1·9
51	30·4	16·3	3·7	2·3
52	24·0	11·0	2·5	1·6

* Commercial, mixed milk. The other samples were from individual cows of the Shinfield herd.

† There is considerable variation in the phosphatase content of the milk from one individual to another. The phosphatase value may be sufficiently low originally in the milk of occasional individual cows (usually in the early stages of lactation) for heating at 61·4(142·5)° to be enough to reduce the blue colour to below the standard of 2·3 units. This in our experience does not occur with the mixed milk of a herd.

They concluded that heating at a temperature 0·8(1·5)° below the required minimum of 62·8(145)° should regularly be revealed by the phosphatase test. As one generally has to deal with mixed milks in commercial pasteurization, it seems probable that this estimate of the sensitivity of the phosphatase test is correct, although data are given for the greater margin of 1·4(2·5)° only.

Jacobsen (59) employing the same method but using a 10 mm. cell and a corresponding standard of 1·8 Lovibond blue units, found similar results in a small number of experiments. In 5 out of 6 samples heated for 30 minutes at 62(143·6)° blue values above 1·8 units were obtained, so that the majority of samples could be distinguished from properly pasteurized milk after being held at a temperature about 0·8(1·5)° below the required minimum of 62·8 (145)°.

Kay and Graham's findings are confirmed by a small number of results reported by Anderson *et al.* (43) and by Trembath and Newman (60).

Koppejan (25) found complete destruction of the phosphatase activity at 62 (143·6)° after even less than 30 minutes' heating. It is likely that the colorimeter which he used in place of the tintometer was hardly sensitive enough to deal with the small amounts of residual phosphatase which are doubtless still active after heating, even for 30 minutes, at this temperature.

For reasons already stated (see page 12) we refrain from a discussion of Storrs and Burgwald's relevant results (38).

Jepsen and Madelung (29) who tested samples which had been heated for 30 minutes at different temperatures below 63(145·4)° found the sensitivity of the test to vary considerably. Whereas in some cases a positive test was obtained after treatment at 61·5(142·7)–62(143·6)°, reduction to 60·5(140·9)° or 61(141·8)° was necessary in others to obtain a reaction indicative of underpasteurization. These variations are difficult to understand, unless the samples used in these experiments consisted of the mixed milk of comparatively few animals. Such samples tend to show greater variability of the residual phosphatase activity than samples bulked in large quantities, as was recently shown by Aschaffenburg and Neave (55). These authors found that, with their modification of Scharer's short test, milk heated for 30 minutes at 61·9(143·5)° generally developed slightly more colour than milk heated for 20 instead of 30 minutes at 62·8(145)°, and even the latter could be clearly distinguished as underpasteurized.

Gilcreas and Davis (12), dealing with pasteurization at a minimum temperature of 61·7(143)° and applying their modification of the Kay-Graham test, found that milk held at 60·6(141)° was easily distinguishable from properly treated samples. This conclusion, based at first on a few experiments only, was amply confirmed by the results obtained from the collective experiment reported by Gilcreas (40), in which samples of the same milks were tested in 11 different laboratories in the United States. The relevant results are summarised in Table II.

TABLE II.

No. of samples	No. of analyses	Heated for 30 min. at	Classified as : pasteurized	under-pasteurized
1	10	60·6(141)°	0	10
2	24	61·1(142)°	2	22
10	110	61·7(143)°	106	4

Burgwald and Gilbertson (39) also found Gilcreas and Davis' modification sufficiently sensitive to distinguish between milks pasteurized at 61·1(142)° and 61·7(143)°.

Thus it appears that the phosphatase test becomes sensitive to a drop in temperature of as little as 0·6(1)°, if applied to milk pasteurized under rather lower temperature conditions than those required in Great Britain. This is remarkable, considering that theoretically the test should become somewhat less sensitive as the temperature requirements are lowered.

According to Scharer (1), Burgwald and Gilbertson (39) and Hahn and Tracy (54), pasteurization for 30 minutes at 61·1(142)° instead of 61·7(143)° is also clearly shown up when Scharer's laboratory test is applied. Jepsen and Madelung (29), however, did not obtain a positive test with regularity in the examination of milk which had been heated for 30 minutes at 61(141·8)°.

From the few relevant data given by Burgwald and Gilbertson (39) and by Roger (56) it appears that Scharer's short test may be almost as sensitive as the laboratory modification. Fay (61), applying Scharer's method (it is not stated whether the short or the laboratory test was used) to milk required to be pasteurized at 61·1(142)°, obtained results indicating that a drop in the pasteurizing temperature of 0·6(1)° was detectable. According to Leahy (57) variations of 0·6(1)–1·1(2)° can also be discovered when using his modification of the phosphatase test.

Summarizing, it may be stated that the phosphatase test, in its appropriate modification, is sufficiently sensitive to detect a drop in temperature of 0·6(1)–0·8(1·5)° if holding for 30 minutes at 61·7(143)° is required, and of about 0·8(1·5)° if the minimum temperature is fixed at the higher level of 62·8(145)°.

(ii) *Sensitivity with regard to holding time.*

This is determined by holding milk samples for periods short of 30 minutes (the time almost universally adopted in the long time—low temperature process) at the minimum temperature required for efficient pasteurization.

Kay and Graham (27), Jacobsen (59), Anderson *et al.* (43), and Trembath and Newman (60) were able to distinguish clearly between samples heated for 20 and 30 minutes at 62·8(145)° or 63(145·4)° when applying the Kay-Graham test. Kay and Graham and Jacobsen tested milk held for 25 minutes as well, but found that only in some cases could it be differentiated from properly pasteurized samples.

Neave (45) obtained similar results with the simplified Kay-Graham test in which the second filtration process is avoided by the addition of sodium hexametaphosphate.

As against these findings Storrs and Burgwald (38) were unable to discriminate between samples heated for 20 and 30 minutes at 62·8(145)°. As has been suggested before, this failure is presumably due to an anomaly in technique, since it is otherwise inexplicable that 9 different samples of milk should all give colour readings below 2·3 Lovibond blue units after heating for only 20 minutes.

Using their adaptation of Scharer's short test to British pasteurizing conditions, Aschaffenburg and Neave (55) found that, as a rule, no difficulty was encountered in classifying mixed milk heated for only 20 instead of 30 minutes at 62·8(145)° as underpasteurized. A small percentage of samples taken from milk bulked in quantities of less than 100 gallons showed a residual phosphatase activity below the rest, but not so low as to lead to a classification of these samples as correctly pasteurized.

Working at a temperature of 61·7(143)° and using their modification of the Kay-Graham test, Gilcreas and Davis (12) found that the majority of samples held for as long as 25 minutes developed a sufficient amount of blue colour to permit their classification as underpasteurized, and they succeeded in the correct identification of 21 out of 22 samples which had been pasteurized for periods from 15 to 25 minutes. The results

obtained in the collective experiment, reported by Gilcreas (40) and summarized in Table III, show however that it is hardly possible to recognise with certainty a reduction in holding time of as little as 5 minutes.

TABLE III.

No. of samples	No. of analyses	Heated to 61·7(143)° for	Classified as : Pasteurized	Under-pasteurized
3	34	15 minutes	0	34
1	10	20 ,,	0	10
7	81	25 ,,	73	8

Gilcreas (42) has drawn attention to the fact that *commercially* pasteurized milk will frequently give a reaction indicative of correct pasteurization after holding for only 25 minutes, since the phosphatase is already partly inactivated during the prolonged period of pre-heating often employed in practice.

Using Scharer's laboratory modification, this author (1), Hahn and Tracy (54) and Burgwald and Gilbertson (39) all obtained results which allowed them to differentiate between samples held for 25 and for 30 minutes at 61·7 (143)°. According to results by Roger (56) a similar degree of sensitivity seems attainable with Scharer's short test, especially when the incubation time is extended to 30 minutes. However, the amount of indophenol colour developed after 25 minutes' heating exceeds the standard permissible for correctly pasteurized milk by so little as to make it doubtful whether a shortening of the holding time by 5 minutes could regularly be revealed by either of these modifications.

It can thus be concluded that the phosphatase test in its various modifications is sensitive enough to detect a reduction in holding time of 10 minutes, but probably not sensitive enough to detect with certainty a reduction of only 5 minutes.

(iii) *Sensitivity as regards the presence of raw in pasteurized milk.*

This is experimentally determined by adding increasing amounts of raw to pasteurized milk. The minimum percentage of raw milk which, without fail, gives colour readings in excess of the value permissible in efficiently pasteurized milk, is here taken as a measure of the sensitivity of the phosphatase test. Since raw milk may vary considerably in phosphatase activity and since even correctly pasteurized milk varies a little in the amount of colour it may give in the test, the addition of still smaller quantities of raw milk will and does, on many occasions, give rise to colours visibly greater than those shown by the corresponding pasteurized sample to which *no* raw milk has been added. We consider that results of this kind provide an unsound basis, at least for present purposes, for the evaluation of the sensitivity of the test.

Using the Kay-Graham test, the percentage of milk necessary to raise the amount of blue colour above the limit fixed for pasteurized milk was found to be as follows :

0·20 per cent. by Kay and Graham (27)
0·25 ,, ,, by Jacobsen (59)
0·25 ,, ,, by Anderson *et al.* (43)
0·20 ,, ,, by Trembath and Newman (60)
0·20 ,, ,, by Jepsen and Madelung (29)
0·10 ,, ,, by Burgwald and Gilbertson (39).

Using their modification, Gilcreas and Davis (12) obtained colour readings suggesting that as little as 0·1 per cent. of raw milk could be safely detected, but the results of the collective experiment (40) given in Table IV, caused Gilcreas (42) to modify this claim.

TABLE IV.

No. of samples	No. of analyses	Raw Milk added	Classified as : Pasteurized	Under-pasteurized
2	21	0·05 per cent.	19	2
3	34	0·1 ,, ,,	23	11

With the same technique, Burgwald and Gilbertson (39) also obtained results indicating that it is necessary to add more than 0·1 per cent. of raw milk to raise the amount of blue colour above the standard permissible for correctly pasteurized milk.

The small amount of information regarding Scharer's laboratory modification makes it difficult to assess the sensitivity of this method. Data by Scharer (1), Tracy and Hahn (53, 54) and Jepsen and Madelung (29) indicate that addition of less than 0·3–0·5 per cent. of raw milk did not always suffice to give a positive phosphatase reaction. According to Burgwald and Gilbertson (39), Scharer's short test does not reveal contamination with 0·1 per cent. of raw milk, but is sensitive to the addition of 0·2 per cent.

Storrs and Burgwald (38) carried out some preliminary experiments on the effect of the addition of raw milk to milk pasteurized at 61·1(142)°. They used the Kay-Graham test with an incubation time of 2½ hours, as recommended for this temperature, and found that amounts of raw milk varying from 0·2 to over 1 per cent. were required to produce more than 2·3 Lovibond blue units. As has been pointed out (see page 12), a reduced sensitivity of the Kay-Graham test to the presence of raw milk is to be expected under conditions requiring a period of incubation of only 2½ instead of 24 hours.

Summarizing, it may be concluded that the phosphatase test will, in general, reveal the presence of 0·2 to 0·25 per cent. of raw milk in the pasteurized product whilst in many cases even smaller amounts will be detectable. Scharer's laboratory modification (and probably the Kay-Graham test also, when adapted to a pasteurizing temperature of 61·1(142)°) seem less sensitive in this respect.

[Note added in proof :

Gilcreas (85) has just reported the results of a collective experiment with Scharer's *short test* similar in arrangement to the collective experiment referred to above. The data show that with the short test it is not possible to detect *with certainty* a reduction of the holding time of 10 minutes or pasteurization for 25 or 30 minutes at 61·1(142)° instead of at 61·7(143)° or the presence of 0·2—0·5 per cent. of raw milk in properly pasteurized samples. Scharer's claim (see page 15) that this test is comparable in accuracy to either his laboratory modification or to the Kay-Graham test cannot, therefore, be upheld, and Gilcreas recommends " (i) that action be deferred on the adoption of the phosphatase field test as developed by Scharer ; (ii) that further studies be made in order that a suitable, precise, and sensitive technique for field use in the control of pasteurization may be developed".]

THE EFFECT OF STORAGE, BACTERIAL CONTAMINATION, Etc. ON THE RELIABILITY OF THE PHOSPHATASE TEST.

Effect of storage.

The remarkable stability of phosphatase *in raw milk* has already been mentioned (see page 6). Scharer (1) found in several instances that milk kept under conditions of refrigeration for periods up to 50 days still showed the phosphatase reaction characteristic of fresh milk. In another case the phosphatase activity decreased gradually with time, but the full activity of raw milk was restored by bringing the pH back to 6·6 through addition of Na_2CO_3 to the sour milk. Thus the enzyme appeared to be still present in its original strength, its action only becoming concealed by the increased acidity of the milk sample. Similar results have been reported by Gilcreas and Davis (47) who observed no change in the phosphatase test when raw milk had been kept for 21 days at 4(39·2)°. Even on storage at 20(68)° no change was found after a period of 7 days, whereas after 10 days a reduction in activity had occurred, possibly caused by souring.

From these results it can be safely concluded that there is no danger of reporting a sample of raw milk as pasteurized, even if the milk has been stored for a number of days before applying the phosphatase test.

Of greater practical importance is the question whether the residual phosphatase activity of treated milk will remain unchanged on storage, in other words, whether correct classification into pasteurized and underpasteurized samples can still be accomplished after such milk has been kept for some time.

Properly pasteurized milks were retested by Jacobsen (59), Anderson *et al.* (43), Gilcreas and Davis (47) and Scharer (1) after periods of cold storage varying from 20 hours to 50 days, and no significant changes were observed. Gilcreas and Davis (12) found, however, on another occasion that after pasteurized milks had been kept for 14–21 days a stronger blue colour developed in the phosphatase test, so that the samples had now to be classified as underpasteurized.

This development of additional blue colour seems to occur after shorter periods of keeping when the pasteurized milk is stored at higher temperatures. Only a few data are available: according to Gilcreas and Davis (47) the blue colour formed in pasteurized milk after 10 days' storage at 20(68)° was still within the range permissible for correctly pasteurized samples, but Anderson *et al.* (43) found the colour increased to the limiting value after the much shorter period of 2 days. The same authors reported an increase of colour far beyond the limiting value when milk had been kept for only 20 hours at 37(98·6)°. It is unfortunate that in most of the experiments just quoted, control tests (*i.e.* without incubation) were not carried out.

The effect of cold storage on *underpasteurized samples* has been studied by Scharer (1) who found that milks which had been pasteurized under conditions short of the required minimum time or temperature, or samples to which raw milk had been added, all behaved similarly to raw milk. The phosphatase activity remained on the whole fairly constant for at least a week, falling thereafter with increasing time of storage. Only after long periods of storage was there any danger that underpasteurized milk would not be revealed as such by the phosphatase test. Adjustment of the reaction of stored milk to a pH of 6·6 led to a partial restoration of the original phosphatase activity. Scharer's findings have been confirmed by Burgwald and Gilbertson (39).

Gilcreas and Davis (47) also observed no significant change in the enzyme activity after underpasteurized milk had been kept for 10 days at 4(39·2)°. A quicker reduction of the phosphatase activity occurred on storage at 20(68)°, but even at this higher temperature the enzyme appeared unaffected by a period of storage of 3 days. Accord-

ing to Aschaffenburg and Neave (55) storage for 2–8 days at 1·7(35)–10(50)° did not affect the results of the phosphatase test, provided the milk was free from (bacterial) taint at the time of retesting.

The majority of the evidence points to the conclusion that the reliability of the phosphatase test is little affected, when samples of milk—whether raw, underpasteurized or pasteurized—are stored for a considerable period under conditions of refrigeration. If, for some reason, it is found necessary to delay testing, samples should always be kept in cold storage.

Effect of preservatives.

A small amount of information is available regarding the effect of preservatives on the phosphatase test. Tracy and Hahn (53, 54) added various preservatives to pasteurized milk and determined the phosphatase activity after 3 days' storage at room temperature and at 7·2(45)–10(50)°. Mercuric chloride showed no effect, borax caused a small, and sodium salicylate and hydrogen peroxide a pronounced increase in the amount of blue colour, whilst formalin reduced the colour development slightly. According to Neave (45) the addition of 1 part of mercuric chloride to 10,000 parts of milk suffices to increase the keeping quality of milk at 20(68)° from one to eight days and has no effect either inhibitory or otherwise on the phosphatase test. Mercuric chloride in higher concentrations was found to inhibit the phosphatase activity in milk to an increasing extent. Recently (81) it has been stated that borax (0·8 g. per 100 ml.) may be added as a preservative to milk which is to be subjected to the phosphatase test by Scharer's technique.

Effect of bacterial contamination.

Kay and Graham (27) pointed out that on rare occasions phenol-producing organisms may develop in pasteurized milk. If control tests are carried out as advocated by these authors an unusually high blue value will, in such circumstances, be found in the controls as well as in the incubated samples so that there would be little danger of classifying such a milk as underpasteurized.

Jacobsen (59), Burgwald and Gilbertson (39) and Neave (45) have investigated the problem of bacterial contamination in more detail. Jacobsen inoculated sterilized milk with pure cultures of several bacteria. Presence of *Str. thermophilus* had no influence on the result of the phosphatase test. Increased blue values were observed when thermo-bacteria, and especially certain strains of hay bacilli, had been added. In actual practice conditions as favourable to the growth of the bacteria as in these experiments are most unlikely to occur.

In similar experiments Burgwald and Gilbertson found that inoculation with various organisms has generally very little effect on the result of the phosphatase test, even if the conditions are so chosen that the different strains are present in abundant quantities. The only organism causing a pronounced increase in blue colour was found to be *Str. liquefaciens*, but it appears most unlikely that under normal conditions in pasteurized milk this strain would develop to a sufficiently large extent.

According to Neave bacteria producing phenolic compounds which react with Folin and Ciocalteu's reagent are not uncommon. *B. cereus*, *B. megatherium*, *B. subtilis* and the green fluorescent organisms frequently found in water are mentioned as examples. After incubation at 37(98·6)° for more than a week, milk artificially inoculated with such organisms gave colour readings equivalent to over 0·4 mg. of phenol per 0·5 ml. of milk.

Other types of bacteria which are a greater potential danger, since their presence is not revealed by abnormally high control tests, are those capable of hydrolysing the

substrate used in the phosphatase test. Neave isolated certain strains (not resistant to heat) of *Bact. coli* and *Bact. aerogenes* which produced colour readings of more than 30 blue units when milk inoculated after pasteurization with 500 million cells per 0·5 ml. was subjected to the phosphatase test (test " B "), provided that no chloroform had been added to the substrate. If the normal procedure of saturating the substrate with chloroform was followed, the colour development, caused in this instance mainly or exclusively by the dead cells, was in general reduced to less than 6 blue units. The hydrolysing substance showed considerable heat resistance, being unimpaired by heating to 80(176)°. It was destroyed by heating for 5 minutes in boiling water. Although contamination of commercially pasteurized milk by coliform organisms occurs not infrequently (62), Neave concluded from many experiments with naturally and experimentally infected milk that bacteria capable of hydrolysing the substrate are unlikely to interfere with the phosphatase test, unless present in sufficiently large numbers to cause the milk to clot on boiling or to produce a (bacterial) taint. If such milk is excluded from testing and if control tests are carried out to reveal the presence of phenol-producing organisms, there appears very little risk that under practical conditions the reliability of the phosphatase test will be affected by bacterial contamination of the pasteurized milk.

Effect of stage of lactation.

As has been mentioned on page 5, the phosphatase concentration in the *raw* milk of individual cows usually reaches a minimum shortly after parturition, after which it increases as lactation progresses. Aschaffenburg and Neave (55) found that the residual enzyme activity of *pasteurized* milk also tends to be low during the first few weeks after calving (see also Table I, p. 16), but does not seem to be influenced by the changes in the raw milk occurring later in lactation. Thus the stage of lactation of the cows contributing milk to a bulk sample is unlikely to affect the reliability of the phosphatase test, unless a bulk sample happens to contain a very high proportion of milk from cows in the early stages of lactation, in which case colour readings indicative of correct pasteurization might be obtained, for instance, after heating for only 20 instead of 30 minutes at 62·8(145)°.

Effect of mastitis.

Owing to the high incidence of sub-clinical mastitis in many dairy herds the possible effects of the changes in the composition of milk caused by this disease on the heat destruction of phosphatase are worthy of consideration. There is little information available on this point apart from a recent investigation by Aschaffenburg and Neave (55) the results of which, although too limited in number to be conclusive, indicate that the phosphatase activity of " mastitis milk " tends to be higher than normal, both before heating and after proper pasteurization.

APPLICATION OF THE PHOSPHATASE TEST TO COMMERCIAL, HOLDER PASTEURIZATION.

Although of paramount importance from the practical point of view, the results obtained from the application of the phosphatase test to the examination of milk commercially pasteurized by the " holder " process are so uniform that a brief discussion will suffice.

The results depend, of necessity, on the standards of sensitivity as determined by the carefully controlled experiments described in the preceding pages. Classification of commercial samples in accordance with these standards does not, by itself,

add greatly to our knowledge, but confirmation of the accuracy and dependability of the phosphatase test has been obtained by the method, much practised by health authorities in the United States and much to be commended, of following up by plant inspection every case in which the phosphatase test indicates faulty treatment. Applying this method, Gilcreas and Davis (47) found that " in practically all instances in which specimens were found improperly pasteurized, the inspection indicated operating conditions which would result in underpasteurization to the degree indicated by the laboratory procedures." These findings were confirmed by Tiedeman (63), Krueger (33), Pincus (64) and Roger (56) and, as their results were based on the application of the phosphatase test to many thousands of commercial milk samples, it may be concluded that the practical application of the test fully corroborates the expectations derived from the carefully controlled laboratory experiments. Interesting accounts are given by each of these authors of the manifold causes of inefficient pasteurization revealed by plant inspection following an unsatisfactory phosphatase test. Krueger points out the special value of the test in enabling supervisory authorities to detect those cases of fraudulent action on the part of the plant operator which would be difficult to discover by plant control alone. According to a more recent publication by Krueger (70), such cases have been practically eliminated in Chicago by strict application of the phosphatase test, and improvements in pasteurizing equipment, particularly in the construction of inlet and outlet valves, have resulted in a further marked reduction of the remaining number of inefficiently pasteurized samples.

Examination of the numerical results obtained by applying the phosphatase test to commercially pasteurized milk emphasises the need for such a test in addition to the customary methods of control. When, in 1935, Kay and Graham (27) first used their improved phosphatase test (test " B ") for the examination of all samples of milk designated as pasteurized which they obtained during a period of six weeks, they found that no less than one quarter of the " pasteurized " milk sold to the public and as much as one half of the " pasteurized " milk supplied to school children was, in fact, either raw or underpasteurized. These surprisingly high figures were confirmed by a survey of unselected samples of milk sold to the public and to schools in London and the South of England, reported by Kay and Neave (65) in the same year. A similar survey carried out two years later by Hoy and Neave (66) showed considerable improvements especially in the percentage of serious errors in pasteurization (Table V).

The improvements were particularly noticeable in milks processed by larger firms having their own control laboratories, the percentage of seriously underpasteurized specimens in these cases falling from 32 in 1935 to 4 in 1937. In spite of the advances in plant design, operating technique and control, conditions were, however, still far from ideal, especially with regard to efficiency of pasteurization in plants belonging to smaller concerns where laboratory supervision was meagre.

Similarly, in 1937, Smith (67) using Kay and Graham's short test (test " A", which is less sensitive than test " B ") for the examination of 218 samples of Ontario milk found 11·9 per cent. to be inefficiently pasteurized.

Gilcreas and Davis (47), from the New York State Department of Health, tested 439 samples of milk and obtained evidence of improper pasteurization in 82 cases (18·7 per cent.). A later report by the same department (63) summarises the results obtained since 1935 from the testing of more than 2,000 samples. As this summary includes the figures for the examination of cream, the data are not strictly comparable, since the position with regard to the application of the phosphatase test to cream appears to be less precise, as will be shown later on. Twenty-nine per cent. of the specimens of milk and cream were classified as being underpasteurized and no less than 8 per cent. gave a phosphatase test indicative of raw milk or cream.

TABLE V.

Comparison of summarized results in 1935 *and* 1937.

Where pasteurized milk sold	Percentage of samples failing to pass the phosphatase test ("B").					
	1935 survey			1937 survey		
	Se.	Mi.	Total	Se.	Mi.	Total
To public in London boroughs *	32	12	44	9	23	32
To public in towns above 20,000 inhabitants (other than London)	17	17	34	11	6	17
To school children in London boroughs ..	33	13	46	0	14	14
To school children in areas other than London	33	25	58	11	11	22
Percentage of total (not average)	29	18	47	8	14	22

Se. and Mi. = Serious and minor errors.

* The percentage quantity of faulty milk in London appears greater than it really is, as a very large proportion of the whole milk is sold by the large firms who now process satisfactorily.
The proportion of serious errors from all sources has diminished from almost one-third to less than one-tenth of the samples examined

Surprisingly low figures were reported by the Department of Health, New York City (64). Examination of more than 10,000 routine samples of milk and cream in 1937 provided evidence of inefficient treatment in only 4·7 per cent. of the cases. A higher incidence of 23·8 per cent. was detected in 1,081 samples collected from suspicious sources. In addition, inspectors of the Department applied the *short* test proposed by Scharer to over 2,000 samples in the field and found evidence of inefficiency in about 10 per cent. of the cases examined.

In a recent survey of 104 pasteurizing plants delivering milk to Rochester, N.Y., Leahy (58) found that 7 out of 157, or 4·5 per cent. of the samples of pasteurized milk examined, gave positive phosphatase tests. In each case gross errors in treatment were discovered by plant inspection.

The figures just related show clearly that efficiency in pasteurization cannot be achieved by plant supervision and bacteriological control alone. The inadequacy of these measures is well summed up in the words of Tiedeman and Hohl (68): "Using standard plate counts for what they may be worth, while making the mental reservation that they have not been worth while, health officials have been forced to depend largely upon the results of inspections to determine the safety of pasteurized milk. Inspections are necessarily infrequent, leaving intervals when almost anything might happen. It is also only natural for plant operators to do their best while an inspector is in the plant, leaving him in "the dark" as to what might happen when he is not there. There has been a great need for a test applied to the final product that will show whether or not it is safe." These authors provide examples illustrating the erroneous results obtained when pasteurized milk is tested by bacteriological methods only.

The relationship between the results of bacterial counts and phosphatase tests has been investigated by other authors. Milligan (69) found agreement between low counts

and negative phosphatase reaction, but whereas the phosphatase test revealed inefficiences in 17 out of 38 samples, only 7 cases produced excessive bacterial counts. Trembath and Newman (60) encountered a number of discrepancies when comparing phosphatase tests with plate counts on lactose agar medium. Further work on a larger scale is reported by Scharer (1). Examination of thousands of samples of commercially pasteurized milk and cream provided evidence that the bacterial counts are influenced by causes other than the degree of efficiency of pasteurization. The author found that low counts do not necessarily mean that the specimen is safe and that high counts occur in cases where the phosphatase test showed the specimen to be correctly pasteurized.

One cannot, of course, entirely dispense with the bacterial examination of pasteurized samples, since the phosphatase test is by its very nature incapable of revealing bacterial contamination introduced after the completion of the pasteurization process.

This possibility was illustrated by Moffat and MacKay (62) who found a positive presumptive test for coliform organisms in 57 out of 100 samples which gave a negative reaction to the phosphatase test. Post-pasteurization infection was indicated by the fact that after re-pasteurization the bacterial test became negative. Similarly, Leahy (58) found that, whereas all samples taken from pasteurizing vats directly after heat treatment were practically free from coliform organisms, a high percentage of samples of the same milks taken after machine bottling was contaminated by these bacteria. Leahy therefore recommends the use of a coliform test in addition to the phosphatase test, concluding that " when both tests are employed simultaneously, they are of more value to the milk industry and public health officials than any other combination of tests yet devised for milk control".

APPLICATION OF THE PHOSPHATASE TEST TO H.T.S.T. PASTEURIZATION.

In Great Britain milk may be sold to the public as " pasteurized " only if it has been processed by the low temperature—long time or " holder " method* and during recent years similar requirements have prevailed in most other countries. The phosphatase test was originally devised in connection with this method and subsequent study and development of the test have been mainly concerned with this form of pasteurization. So far discussion has, therefore, been limited to this aspect.

An alternative way of pasteurizing milk, heating at higher temperatures (71(160)–75(167)°) for much shorter " holding " periods (10–20 seconds), already permissible in the United States by the 1936 Milk Ordinance and Code of the Public Health Services, is receiving increasing attention and the question whether the phosphatase test remains applicable and sensitive under such high temperature—short time (H.T.S.T.) conditions is of increasing importance.

It is somewhat outside the scope of this bulletin either to dwell on the respective merits and demerits of the two methods of pasteurization or to endeavour to influence a decision for or against either of them. The fact that, as recently reported by Krueger (71), a milk centre of the importance of Chicago contemplates admission of the H.T.S.T. method as a recognised alternative to the " holder " process must, however, be quoted. The contamination of " holder " equipment with thermophilic organisms has greatly influenced the Chicago health authorities in adopting this policy. When " holder " equipment is used for hours on end, the development of bacteria of this type is greatly favoured and, once they have infected part of the plant, decontamination becomes a

i.e. " retained at a temperature of not less than 145° and not more than 150° Fahr., for at least 30 minutes and be immediately cooled to a temperature of not more than 55° Fahr." (Milk (Special Designations) Order 1936. S.R. & O. 356).

difficult problem. According to Mattick and Hiscox (72), this serious trouble is unlikely to occur under H.T.S.T. conditions. The H.T.S.T. process has, in fact, been used to replace temporarily the " holder " process in a plant completely incapacitated by spore-forming thermophiles (73).

For this and other reasons it appears that, as developments in plant design are leading to greater accuracy and safety in the processing of milk by the H.T.S.T. method, this alternative will steadily gain in importance and may in the near future lead to far-reaching changes in the existing regulations. With this likelihood in view the question of the applicability of the phosphatase test to H.T.S.T. pasteurization has received some preliminary attention.

The amount of work carried out in this direction is small when compared with that which has been done on the value of the test as applied to " holder " pasteurization, and that for two reasons: first, that the " holder " process, as the main " legal " process, is obviously of major importance and second, that the evaluation of the sensitivity of the phosphatase test under H.T.S.T. conditions presents many serious experimental difficulties. Whilst it is easy to imitate " holder " pasteurization in the laboratory and thus, for instance, to prepare samples underpasteurized to a known degree, the problem of accurately heating small samples of milk at known temperatures for exact periods of a few seconds only, appears so far not to have been solved. Laboratory apparatus of the kind described by Storrs and Burgwald (38), based on controlled flow through glass tubes, though giving useful data, cannot be regarded as providing even an approximately exact counterpart of the commercial H.T.S.T. method.

Those who have studied the applicability of the phosphatase test to H.T.S.T. pasteurization have, therefore, mostly contented themselves with the testing of samples obtained from large scale pasteurizers. As large quantities of milk are thus involved, few opportunities arise to conduct planned time and temperature trials necessitating alterations in the routine to which the plant in question is working. Even if such facilities are granted, it is not easy to arrive at conclusions of general validity, as the results are bound up with the type and lay-out of the plant used. This applies in particular to all cases in which pasteurizers are employed which are not built to standard. Mattick and Hiscox (72) have, for instance, pointed out that in some types of Stassano machines pipes of varying length and diameter are inserted between the tubular heater and the plate heat exchanger, causing the milk to be held at high temperatures for a variable period in addition to the specified time of heating. This additional period must be taken into consideration before generalizing from results obtained with one particular plant.

A fundamental difficulty remains in every case—the impossibility of assessing the true period during which the milk is actually exposed to the effective temperature of pasteurization. This period is doubtless different from the heating time as calculated from the rate of flow and the dimensions of the plant, since the milk entering the heating section from the preheater will require some time to attain the maximum temperature of pasteurization. In endeavouring to obtain samples the treatment of which is exactly known, for the determination of the sensitivity of the phosphatase test under H.T.S.T. conditions, the margin between the true and the calculated period of heating thus remains as a factor of uncertainty even under the most favourable circumstances.

The task of comparing the data presented by various authors is complicated by their lack of uniformity in describing the heat treatment of the milk samples. When using apparatus of the type containing specific heating and " holding " or " retarding " sections, as for instance A.P.V. plate heaters, reference is frequently made to the " holding time " only, without mention of the " heating time". As pasteurizing effects will already take place in the heating section in which the preheated milk is brought to the holding temperature, results based on " holding time " only cannot be

compared with those obtained from samples pasteurized in apparatus of the type containing no separate holding section, as for instance the "cylinder" design of Stassano, since of necessity the "heating time" here refers to the total time required for the milk to pass from the preheater to the cooling section. As far as possible the available data will, therefore, be dealt with in sections concerned with milk pasteurized in apparatus of the same type.

Kay and Graham (27) were the first to apply the phosphatase test to H.T.S.T. pasteurized milk. In 14 samples from different runs of the same Stassano machine of the "cylinder" design (milk maintained at 75(167)° for a period believed to be between 15 and 20 seconds) the test was found to be uniformly negative. This was confirmed by Trembath and Newman (60) using a pasteurizer of the same type (time of heating 18–20 seconds). In both cases no indication is given of the length of pipe between heater and regenerator, so that the additional holding period cannot be ascertained. Using a machine in which this additional holding period amounted to 7 seconds Mattick and Hiscox (73) found practically complete destruction of the phosphatase in 46 cases in which the milk had been heated at 73·9(165)–75(167)°. Unfortunately, few data are available showing the effect of a further reduction in temperature or time of heating on the phosphatase activity of milk pasteurized in this type of apparatus, except for a single observation by Trembath and Newman (60) who obtained a positive phosphatase test at 72·2(162)° and an investigation by Winther (74) showing that the test became positive at temperatures below 72(161·6)°. These data are insufficient to evaluate the sensitivity to underpasteurization in an accurate manner, but from other evidence it appears that the test loses some of its sensitivity if it is applied to pasteurization at a temperature as high as 75(167)°. It was found (74) that the phosphatase test became negative when milk was heated to just above 72(161·6)° in 1·4 seconds and held at this temperature for 5·4 seconds only, using the much improved 1937 Silkeborg Stassano plant. This means that with a total time of heat treatment of the same order (approximately 7 seconds) as the time of holding alone in the experiments reported by Mattick and Hiscox the phosphatase test still remained negative at a temperature about 3(5·4)° below that required in the older type of Stassano apparatus. It seems reasonable to conclude that only fairly gross errors in time and temperature of pasteurization will be detectable by the phosphatase test, if it is used for milk which has been processed in pasteurizers designed for treatment at 75(167)° for comparatively long periods (approximately 20 seconds).

Fortunately, the test becomes more sensitive at the lower temperatures which are nowadays more generally favoured. Short time pasteurization at temperatures between 71·1(160)° and 72·2(162)° is gaining more and more in importance, as it is realised that the milder treatment affects the commercial properties of milk (cream line, etc.) to a lesser degree whilst still remaining effective in destroying the pathogenic flora, provided the milk is *held* at these temperatures for an adequate length of time. The importance of a short but definite holding time, when working at these temperatures, cannot be over-emphasized. Mattick and Hiscox (73) have shown that milk heated to 72·2(162)° in approximately 11 seconds and cooled immediately without further holding still contains considerable amounts of phosphatase (12–21 Lovibond blue units), whereas the enzyme as mentioned above, is practically completely destroyed when milk is heated to 72(161·6)° in 1·4 seconds and held at this temperature for only 5·4 seconds (74).

Data obtained by testing milk pasteurized at 71·1(160)° or 71·4(160·5)° with a holding time of 15–18 seconds have established the fact that a negative phosphatase test results under these conditions (1, 12, 39, 71). Regarding the minimum reduction in holding time or temperature required to render the phosphatase test positive, little

information is available outside the recent results of Mattick and Hiscox (73). In a lengthy series of experiments with a commercial pasteurizer milk was heated in approximately 15 seconds and held for 11·5 seconds at temperatures which varied from experiment to experiment. A negative phosphatase test was obtained whenever pasteurization took place at 71·1(160)° or above, whilst the test became positive as soon as the holding temperature fell to 70·8(159·5)° or below. Simultaneous experiments showed that no tubercle bacilli survived, even if pasteurization took place at 70(158)°. These findings confirm the important conclusion (see p. 7 and Fig. II) that the phosphatase test applied to milk pasteurized by the H.T.S.T. process begins to indicate underpasteurization under conditions which could still be regarded as safe from the hygienic point of view, *i.e.* the phosphatase test under H.T.S.T. conditions no less than under " holder " conditions has a margin of safety from the public health stand-point. In order to increase this margin of safety, Mattick and Hiscox recommend pasteurization at 72·2(162)° with a holding time of 15 seconds and, similarly, Krueger (71) reports that in Chicago holding for 16 seconds at 71·7(161)° is now demanded. By raising the requirements in accordance with these proposals, one nevertheless reduces the sensitivity of the phosphatase test, as it has been shown that a holding time of 11·5 seconds at 71·1(160)° suffices to destroy the phosphatase. Further evidence is needed before deciding whether it is preferable to adhere to the increased margin of safety now in favour or to adopt less severe standard conditions, say holding for 15 seconds at 71·1(160)°, which would make for an increased sensitivity of the phosphatase test and would thus allow health authorities to exercise a stricter control.

Little need be said about the remaining data concerning H.T.S.T. pasteurization. Hoy and Neave (66) and Mattick and Hiscox (73) applied the phosphatase test to samples pasteurized in older types of apparatus (tubular and Danish rotary heaters). Since the duration of heat treatment could not be ascertained with any degree of accuracy and as considerable fluctuations occurred during processing, only the broadest conclusions could be drawn from the rather inconsistent results. In a number of Danish investigations (75), on the other hand, the performance of several types of modern H.T.S.T. pasteurizing apparatus was accurately assessed, but Stein's modification of the phosphatase test was employed. This, as has been mentioned before (see page 9), has been found lacking in sensitivity by Jepsen and Madelung (29). These latter authors worked with commercially heated milk (Stassano process) whose heat treatment was not known in detail, but obtained results indicating that the phosphatase test is likely to remain an accurate means of control when applied to milk pasteurized by the H.T.S.T. process.

Gilcreas and Davis (12) and Spencer (37) obtained negative phosphatase tests for milk pasteurized in the laboratory for 15 seconds at 71·1(160)°. No indication is given of the technique used in preparing the samples. The results obtained by Storrs and Burgwald (38), in the glass apparatus already referred to, vary considerably : using an incubation time of 24 hours in the Kay-Graham test, samples held for 15 seconds required temperatures between 70·8(159·5)° and 72·2(162)° to render the phosphatase test negative. It has been shown above that samples of milk after large scale pasteurization at 71·1(160)° with 15 seconds' holding invariably gave a negative test and it appears, therefore, that the inconsistencies found by Storrs and Burgwald are caused by fluctuations in the performance of the laboratory pasteurizer.

The sensitivity of the test to the addition of raw milk remains, of course, the same under H.T.S.T. as under " holder " conditions, since it does not depend on the heat treatment of the sample.

To sum up, the claim that the phosphatase test remains sensitive under H.T.S.T. conditions is well founded, but further experimental evidence is required to settle a number of details. The test is undoubtedly very delicate in the time-temperature range which is critical as regards the destruction of pathogenic micro-organisms,

but its sensitivity diminishes rather rapidly when the standard requirements for pasteurization are increased, either for temperature or for time, above 71·1(160)° and 15 seconds. Insufficient evidence is at present available to enable us to state precisely what holding *time* at this temperature will first lead to a phosphatase test indicative of underpasteurization.

APPLICATION OF THE PHOSPHATASE TEST TO PRODUCTS OTHER THAN MILK.

The published evidence is practically unanimous that for milk, where conditions of carrying out the test have been worked out with great care, the phosphatase test is surprisingly delicate and effective. Attempts have been made to apply the test to other dairy products, such as skim milk, butter, cheese and, in particular, to cream. Unfortunately, with these products, it has not always been used in a judicious manner. The logical procedure, such as was followed in the working-out of the phosphatase test for milk, of basing the technique of the test on a careful preliminary examination of products of accurately known thermal and other history, has up to now been somewhat neglected. It is, therefore, not surprising that the accuracy with which the test may be applied to products other than milk is still disputed and careful laboratory investigations are required to place the test on a more satisfactory basis.

Cream.

In particular does this apply to cream. In the United States the phosphatase test has been extensively used in controlling the efficiency of cream pasteurization. Some workers (1, 33, 47, 52, 63, 64) have employed the technique as evolved for milk without any alteration whatsoever, thereby tacitly assuming that the amount of residual phosphatase in correctly pasteurized *cream*, *viz.* cream pasteurized under the minimum conditions required by regulation, will never be greater than the maximum amount permissible for correctly pasteurized *milk*. There are strong grounds for believing that this assumption is not correct.

When Kay and Graham (15) first proposed the phosphatase test, they observed that the enzyme is not evenly distributed in milk, but is preferentially associated with the fat globules. Raw cream was found to contain a far higher concentration of phosphatase than the milk from which it had been prepared, an observation recently confirmed by Gilcreas (42). In spite of this the enzyme appeared to be completely destroyed after laboratory pasteurization of the cream under the standard conditions laid down by the Ministry of Health (Great Britain). It must, however, be remembered that the test which was used in Kay and Graham's early (1933) experiments is not very sensitive to small amounts of residual phosphatase.

With the help of the more sensitive modifications of the phosphatase test introduced in 1935 and subsequently it has been shown that correctly pasteurized milk may contain small, but measurable, amounts of residual enzyme. It is reasonable to assume that the phosphatase in raw cream, greater in amount per unit volume than that in the milk from which the cream was derived, will be not less thermostable than the enzyme in milk. If this is true, we should expect rather more phosphatase per unit volume to be left after the pasteurization of cream than after the pasteurization of milk. It has already been shown that the destruction by heat of the phosphatase in milk follows the course of a monomolecular reaction (see page 6) so that, provided the reaction constant is the same for cream as for milk, the amount of phosphatase remaining active after a given time of heating at a given temperature will be proportional to the original concentration of the enzyme which in cream is much higher than in milk. Fay (61) has actually found that when skim milk, milk containing

4 per cent. of fat and cream containing 20 and 40 per cent. of fat were prepared from the same whole milk and heated under identical conditions, "the phosphatase was destroyed at lowest temperatures and in shortest times in the skim milk, and that it required progressively and correspondingly more temperature and time with each increase in the fat content of the sample". Since it is one of the bases for the sensitivity of the phosphatase test that a slight increase in the amount of enzyme left after pasteurization leads to large effects, the enzyme concentration—phenol concentration curve rising very steeply over the range of small enzyme concentrations (see page 10), the colour readings obtained with correctly pasteurized cream should be significantly higher than those obtained with correctly pasteurized milk.

Trembath and Newman (60) have, in fact, observed "that heating cream and milk in exactly the same manner, to the same degree, and for the same time, that is, at 62·8(145)° for thirty minutes, invariably resulted, upon application of the test, in the production of a blue colour from the cream greater than the 2·3 units standard, while the milk registered colours less than 2·3 units". This was found in each of 20 samples of cream.

Another contributory cause to the higher phosphatase figures for properly pasteurized cream may be, as Fay (61) points out, the higher viscosity of cream. This would probably result in slower heat penetration and hence in a shorter time of effective exposure to heat than the apparent time of heating.

Colour readings for pasteurized cream from 2 to 10 times in excess of the standard permissible for correctly pasteurized milk have also been reported by Gilcreas and Davis (47) (in Table VIII of their paper), but it would appear that in routine testing these authors have nevertheless continued to treat cream as if it were milk. Their report on the examination of 780 routine samples gives the percentage of samples found improperly pasteurized as 18·7 for milk and as 38·2 for cream. The high incidence of cream samples failing to pass the test may not have been due to a reduced efficiency in the pasteurization of cream, but to the application of a limiting blue value which, while satisfactory for milk, appears to be too low where cream is concerned. Since in commercial practice cream will frequently be processed under conditions exceeding the minimum requirements for time and temperature of heating, a good many pasteurized cream samples will give a negative phosphatase test and one may easily be misled into concluding that no change in the technique of the phosphatase test is required when cream is tested instead of milk.

It is possible, however, that Gilcreas and Davis' findings have been caused by another closely associated factor to which attention has been drawn recently (42, 52). Gilcreas (42) showed in some detail that cream, separated *after* pasteurization from milk giving a negative phosphatase test, frequently produces colour readings exceeding the maximum permissible for correctly pasteurized milk. This applied to cream separated by gravity as well as by mechanical means. Thus 25 per cent. of a series of 139 samples of cream obtained from milk adequately pasteurized under commercial conditions (and giving a negative phosphatase test) gave reactions indicative of under-pasteurization. This observation is quite in line with the findings of Kay and Graham concerning the accumulation of the enzyme in raw cream.

In view of these findings there can be little doubt that some modifications in technique are necessary in order to use the phosphatase test as a just and sensitive method of controlling the efficiency of cream pasteurization. It seems unlikely that this can be achieved by simply raising the colour standard, since cream may vary considerably in composition and since a close relationship undoubtedly exists between butter fat content and phosphatase activity. If it can be proved that these properties are fairly closely proportional to each other, then the alteration proposed by Kay and Neave (44), *viz.* to dilute the cream with water to a butterfat content of 4–6 per cent., and then to treat it as if it were milk, would appear to overcome most of the

objections which must be raised against the method of applying the phosphatase test to undiluted cream in the same manner as to milk. The drawback of the dilution method is that it will reduce the sensitivity of the test to contamination by raw milk. This sensitivity has been shown to remain of the same order as with milk, when raw milk is added to pasteurized cream and the mixture tested as if it were milk (40).

Parfitt and Brown (76) have recently shown that the pH of the buffer mixture used in the phosphatase test may be reduced below the range of optimal enzymic activity if cream, and especially sour cream, is tested like milk. The danger of obtaining erroneous results from this cause would be avoided by diluting the cream in the manner suggested by Kay and Neave.

The effect of the pH of cream on the enzyme itself requires investigation. Mattick (77) has pointed out that even ordinary micro-organisms in milk or other products become more resistant to heat as the pH rises and that it is, for instance, necessary to heat neutralized cream to a higher temperature than milk in order to bring about approximately equal bacterial destruction. The thermal stability of phosphatase may be influenced similarly by the pH of the cream.

The effect of storage on the phosphatase in cream seems to be similar to its effect on the phosphatase in milk. The enzyme activity is reduced if raw cream is kept at room temperatures for periods of four days to a week (76). Prolonged storage of pasteurized cream at 4(39·2)° and 20(68)° gave erratic results in experiments reported by Gilcreas and Davis (47) who put the discrepancies down to the butyric acid fermentation of the cream. There appears little danger that storage of cream for a limited period will affect the accuracy of the phosphatase test.

Finally, attention must be drawn to the need for studying the application of the phosphatase test to cream pasteurized by methods other than the " holder " process.

According to Scharer (52) a strongly positive phosphatase test is obtained when cream is pasteurized for 15 seconds at 71·1(160)°, *viz.* under conditions which lead to a negative test with milk, but it remains to be seen whether such cream diluted according to the suggestion of Kay and Neave will give the same result. Shadwick and Parker (78) report that heating for 2–3 seconds at a temperature as high as 85(185)° is necessary to reduce the colour with undiluted cream subjected to the 24 hour test of Kay and Graham to 2·3 blue units or below, this standard being exceeded when the temperature is reduced to 82·2(180)°. These figures suggest that the enzyme is less easily destroyed in cream than in milk, if the " flash " process is applied. Considerations of viscosity and the rate of heat penetration will obviously apply with greater force to the pasteurization of cream by the H.T.S.T. or " flash " process than to its pasteurization by the " holder " method.

Although investigations of the stability of the phosphatase in cream to the times and temperatures and other conditions pertaining to H.T.S.T. and " flash " processing are, therefore, most desirable, it seems even more urgent to study the destruction of *pathogenic* micro-organisms in cream under these conditions, about which hardly anything appears to be known. There is no point in devising methods for applying the phosphatase test to H.T.S.T. or " flash " pasteurized cream, before it has been ascertained whether it is safe to process cream under the same minimum conditions as milk or whether these conditions ought to be raised in order to retain a reasonable margin of safety.

Skim milk, etc.

From the fact that there is a greater concentration of phosphatase in cream than in milk it follows that the enzyme content of skim milk must be lower than that of whole milk. For raw skim milk this has been verified by Kay and Graham (15), and Gilcreas (42). With skim milk derived from commercially pasteurized whole milk, Gilcreas obtained colour readings which were not significantly lower than those of

the whole milk, but as the whole milk had, in all likelihood, been processed under conditions exceeding the minimum requirements, these results cannot be accepted as conclusive. Further experiments with skim milk accurately pasteurized under laboratory conditions or separated from accurately pasteurized milk are needed to show whether it would be advantageous to introduce a lower limiting standard of colour in order to avoid the loss in sensitivity which will probably result if skim milk is treated as if it were whole milk.

It has further been claimed that the phosphatase test is applicable to goat's milk (33, 52), soft curd milk (33), vitamin D milk (33, 52), and condensed milk (52). Krueger (33) finds the test not wholly satisfactory for chocolate or cocoa milk or beverages, whereas Scharer (52), using his modification instead of the Kay-Graham test, states that chocolate milk can be tested without difficulty. As pointed out by Scharer the carrying out of control tests is of particular importance when dealing with products of this kind.

Butter and buttermilk.

Kay and Graham (15) observed that butter made from raw cream contained considerable amounts of phosphatase and that, moreover, the aqueous layer obtained by melting and centrifuging such butter was so rich in enzyme that samples had to be diluted ten to twenty times in order to make a convenient determination of the phosphatase content possible.

As in the case of pasteurized cream, no appreciable phosphatase activity could be detected in butter made from such cream on application of the 1933 phosphatase test. Since, however, the enzyme is even more concentrated in butter and especially in butter serum than in cream, it is again only reasonable to assume that measurable amounts of residual enzyme are present in butter made from correctly pasteurized cream and that they may, in fact, be detected by the more sensitive modifications of the phosphatase test now in use. Consequently, all the arguments discussed when dealing with cream must be considered again, if, on testing butter, it is desired to attain a degree of precision similar to that obtained when applying the test to milk or cream. This precision has never been attempted by Kay and collaborators (15, 27, 44) who were satisfied to show that the qualitative (short) modification of either their original (1933) or their improved (1935) phosphatase test could be employed to distinguish between butters made from raw and from heated cream. For this purpose they proposed to use the technique evolved for milk, replacing the milk by butter serum prepared either by simply melting and centrifuging butter or by adding water to butter contained in a large test tube in the proportion 1 : 2, melting and cooling the stoppered, inverted test tube in the refrigerator until the fat had solidified. Negative (short) phosphatase tests were obtained with butter made from " holder " pasteurized cream and with butter made in New Zealand from " flash " pasteurized cream, whereas butter prepared at the same time from raw cream showed a marked reaction, even after cold storage over a period of six weeks.

Recently Scharer (52), and Parfitt and Brown (76) have applied the modification of the phosphatase test developed by the former to the examination of butter, and Shadwick and Parker (78) have used this method as well as the (long) Kay-Graham test. No notable changes in technique have been made, milk being again simply replaced by an equal amount of butter serum.

Parfitt and Brown observe that serum of butter made from sour cream reduces the pH of the buffer substrate mixture on occasion, just like sour cream itself, below the range of optimum activity of the phosphatase. This difficulty may be avoided either by diluting the butter serum or by using less butter serum than milk, procedures which in view of the fact that phosphatase tends to accumulate even more strongly in the aqueous butter layer than in cream, appear sounder than the application of the unaltered technique.

They also draw attention to a number of other difficulties which may complicate the phosphatase test when dealing with butter. Thus, the water used for washing butter is sometimes chlorinated, which affects the colour development with Gibbs' reagent. A considerable reduction of the phosphatase activity takes place when butter is washed and salted. Other difficulties (which can, however, be dealt with by carrying out control tests) are contamination of the washing water with phenol and also occasional bacterial contamination.

Shadwick and Parker have tested starters used for buttermaking, lime and soda used for neutralizing sour cream and salt used for salting butter, and have found that none of these agents interfere with the phosphatase test. These authors have observed that butter which gives a negative phosphatase test before being subjected to the keeping quality test is liable to give reactions indicative of underpasteurization when it is examined again after storage for 8 days at 21·1(70)°. The same applies to butter *serum* kept under identical conditions, but not to butter serum to which chloroform has been added or which has been kept at 5·6(42)°. Phenol production by bacterial development is, therefore, indicated, but unfortunately not proved by carrying out control tests.

Shadwick and Parker have found that butter freshly prepared from neutralized cream gives a positive phosphatase test unless the cream has been " flashed " for 2–3 seconds at temperatures of 85(185)° or above. Parfitt and Brown have examined 612 samples of commercially pasteurized butter of which 185 have been found to develop more colour than is permissible for correctly pasteurized milk. This standard is undoubtedly too severe when dealing with butter, but it is remarkable that on analysis the majority of the " underpasteurized " samples are found to have been prepared from " flash " pasteurized cream. Scharer observes similarly that butter prepared in this manner shows " considerable " activity whereas butter made from " holder " pasteurized cream is found to give only " slight " activity. It appears at present impossible to distinguish a butter made from " correctly " " flash-pasteurized cream " from a sample made from slightly underpasteurized cream processed by the " holder " method. This again emphasises the need, already commented upon, for further study of the problems connected with the H.T.S.T. and " flash " pasteurization of cream. In the meantime, the phosphatase test as applied to butter must—as Parfitt and Brown point out—be regarded rather as a useful tool than as an instrument of precise control.

Little information is available with regard to buttermilk. Kay and Graham (15) applied the original phosphatase test to buttermilk from raw and pasteurized creams. Almost as much phosphatase was found in buttermilk from raw cream as in the cream itself whereas buttermilk from pasteurized cream showed no enzyme activity. On the other hand, Jacobsen (59), applying the improved Kay-Graham test, obtained colour readings indicative of underpasteurization (according to the standard for correctly pasteurized milk) when testing buttermilk, " although the samples were ' flash ' pasteurized and thus Storch negative". Jacobsen suggests the production of small amounts of phosphatase by the lactic acid bacteria as a possible cause of the high colour readings.

Cheese.

Kay and Neave (44) stated that cheese made from raw milk contains considerable amounts of phosphatase for at least two months after manufacture. Scharer (52) tests cheese by mixing 20 g. of cheese intimately with 20 ml. of buffer and an equal amount of water ; 1 ml. of this mixture is then submitted to the phosphatase test in the usual way, as if it were milk. No reduction in enzyme activity of cheese made from raw milk occurs after as much as 18 months' storage whereas cheese made from

"holder" or "flash" pasteurized milk shows no phosphatase reaction. Cheese prepared from underpasteurized milk or cream gives a positive reaction. Examination of commercial samples shows that some types of cheese give positive phosphatase tests more often than others. Control tests should be carried out in the usual manner. With some types of cheese coloured filtrates are obtained, making the colour reading difficult. This difficulty may be overcome by extracting the indophenol colour formed with Gibbs' reagent into butyl alcohol and comparing the depth of colour in the butyl alcohol with that produced by carrying out the same operation on a sample of cheese known to give a negative reaction.

It thus appears that the phosphatase test can be usefully applied to the examination of cheese. It would be superfluous to insist on the same degree of precision as is necessary when the test is used for milk or cream.

Ice Cream.

Recently the possibility of applying the phosphatase test to ice cream has been studied by a number of authors (33, 50, 51, 52, 79, 80) in the United States, where this product is of far greater importance than in most other countries. Many difficulties have been encountered owing to the almost unlimited variety of ingredients used in the composition of ice cream mixes.

In order to study the effect of time and temperature of heating and of other factors in the clearest possible manner, Hahn and Tracy (79) and Caulfield and Martin (80) made up standard mixes from raw cream, raw skim or whole milk, condensed or dried milk, sugar and gelatine without addition of flavouring or colouring matter. Standard mixes of almost identical, composition were used in both investigations, containing 12 per cent. fat, 10 per cent. serum solids, 15 per cent. sugar and either 0·3 or 0·35 per cent. gelatine. When these were heated for 30 minutes at 62·8(145)°, they were found to contain considerable amounts of residual phosphatase. Heating at 65·6(150)° or thereabouts was required to reduce the enzyme content below the maximum amount permissible for correctly pasteurized milk. Caulfield and Martin were able to show that the increased resistance of the enzyme at temperatures below 65·6(150)° is caused by the protective action of the sugar. When mix was prepared without incorporation of sugar, heating for 30 minutes at 62·8(145)° or even at 61·1 (142)° sufficed to destroy the enzyme in the mix to the same degree as in milk. According to Scharer (51), the pasteurizing conditions for ice cream laid down by American health authorities are more severe than those pertaining to milk. Thus the U.S. Public Health Service suggests heating for 30 minutes at a minimum temperature of 65·6 (150)°, whilst the sanitary code of the City of New York even requires heating for the same period at 68·3(155)° when raw products are used for mixing. Under these conditions the phosphatase test in its present form seems to lose much of its sensitivity to minor faults in pasteurization. Caulfield and Martin (80) are, for instance, unable to classify with regularity as underpasteurized ice cream mix heated for only 15 or 20 instead of 30 minutes at 65·6(150)°. Alterations in technique will, therefore, be necessary to increase the sensitivity of the test and Scharer suggests an extension of the incubation time for this purpose. With the Kay-Graham test, or its modification by Gilcreas and Davis, this procedure is hardly practicable, as normally a period of incubation of 24 hours is already employed. But even without alterations in technique the phosphatase test remains, of course, as sensitive to contamination with raw milk or cream as in the testing of milk. This is of importance, since standardization of pasteurized mix with raw products is one of the most common faults encountered in practice (51, 52).

Storage of ice cream for 2 weeks at 4·4(40)° (79) or at about −12·2(10)° or −23·3(−10)° (80) does not appreciably alter the result of the phosphatase test, whilst

variable results are obtained on storage for longer periods. Homogenization or freezing are practically without influence (80). Interference is frequently caused by the presence of colouring and flavouring materials normally incorporated into ice cream mix. Food colours are apt to interfere if they are not precipitated with the proteins, and difficulties arise in extracting the indophenol colour into butyl alcohol when Scharer's short method is employed. Of the many flavouring ingredients examined, pure vanilla has little effect unless used in excess ; artificial vanilla products may give rise to additional colour development ; vanillin and, in particular, coumarin increase the amount of blue colour formed in the test. Raw fruits, synthetic fruit flavours, and fruit syrups may contain or produce compounds which react with the phenol reagent and it is, therefore, absolutely essential to carry out control tests whenever a positive test is obtained with ice cream mix. Ingredients may also be incorporated which contain a phosphatase, such as lemon juice or certain unroasted nut meals (51).

In view of all these difficulties the results obtained by applying the phosphatase test to ice cream mix require careful interpretation and, as with butter, the test must for the present be regarded rather as a useful tool than as an instrument of precise control.

SUMMARY AND CONCLUSION.

Six years have now elapsed since the publication of the first, relatively undeveloped form of the phosphatase test, and only four since the more sensitive method was first put forward. In these six years the test has developed into what has been described as the most useful weapon for controlling the efficiency of " holder " pasteurization yet devised.

The basis for this development is the fortunate combination of properties which this enzyme possesses in relation to milk. It is present in reasonably large quantities in all samples of raw milk ; it remains practically unchanged for many hours in unheated milk, and for days if this milk is kept cold ; its thermal stability is such that it is very nearly completely destroyed by exposure of the milk to the minimum temperature and time requirements for " legal " pasteurization ; it is rather more resistant to heat than the common pathogenic organisms which may occur in raw milk ; it is, in particular, slightly more resistant to heat over all ranges of time and temperature of heat exposure than is the tubercle organism ; it can be detected and estimated in raw or heated milk in very small quantities ; its detection and estimation in small quantities, though requiring a certain degree of laboratory skill and experience, is not difficult and needs neither elaborate apparatus nor complicated technique.

In connection with H.T.S.T. pasteurization, experience of the value of the phosphatase test is still limited, but there can be no doubt that, possibly with minor modifications, it will provide as sensitive and searching a method of control here as it has already provided for " holder " pasteurization.

The phosphatase test for " holder " pasteurization, in its accurate form, has only been in use some four years. Although it is perhaps too much to expect that complete legal or official recognition would be extended to a test, however satisfactory, in such a relatively short time, official comment in Great Britain during this time has not been unfavourable. In 1935 the Chief Medical Officer of the Ministry of Health (England and Wales) stated that the test " may now be regarded as an important means for the control of pasteurization " (82) and, again, in 1936 : " The phosphatase test is a valuable supplement to inspection of the plant and a positive result affords convincing evidence that pasteurization has not been efficiently carried out. The obligation to detect in what way the pasteurization plant or technique is at fault actually rests with the licensee, and if he cannot deliver milk which satisfies the requirements of the phosphatase test he should not continue to hold a licence." (83).

In view of the great amount of favourable evidence accumulated since these statements were made, there seems to be an excellent case now for official recognition of the test, at least as far as the control of " holder " pasteurization of milk is concerned, which is the only legally approved heat treatment in Great Britain. It has been proposed that countries permitting H.T.S.T. pasteurization as well, should lay down no temperature or time conditions for pasteurization, but simply insist that in heat-treated milk the phosphatase test, carried out under closely defined conditions, shall be negative, and the heating plant shall be otherwise satisfactory to the inspecting authority. This seems worthy of serious consideration and in fact has been under discussion recently in certain Scandinavian countries. There would appear to be no serious criticism of this quite simple and yet, as all experience goes to show, quite effective stipulation. The increasing interest of consumers in the cream line would prevent overheating.

In the United States various modifications of the 1935 test are on trial. The Association of Official Agricultural Chemists has adopted Gilcreas and Davis' modification of the Kay-Graham test as a " tentative " method (41) and has just completed a study of Scharer's short modification of the test (81) (see page 20). The American Public Health Association has reported that " in the near future this association should

appoint a special committee to study the phosphatase test and suggest modifications with the purpose of proposing a standard procedure for adoption by the American Public Health Association as an official test for the pasteurization of milk" (84). For maximum efficiency and usefulness of such a standard procedure, standard requirements for holder pasteurization (and for H.T.S.T. pasteurization also) applicable to the whole country would be almost essential. At present official pasteurization requirements appear to vary appreciably in different parts of the United States.

In reviewing the present status of the phosphatase test we have endeavoured to draw attention to the various problems still awaiting solution, particularly those regarding products other than milk, in the hope that this course will stimulate further investigations. These should be concerned not only with the behaviour of the enzyme and the phosphatase test itself, but also with closely related questions such as the thermal destruction of pathogenic organisms in dairy products other than milk, about which relatively little is known. Only when the time and temperature required for complete destruction of possible pathogens in any particular milk product have been determined with accuracy, will it be possible to lay down safe and suitable minimum requirements for its pasteurization, and not till then will it be possible to make the phosphatase test as accurate for that milk product as it is for milk. Owing to the many possible variables in technique (amount of material to be used for testing, time of incubation, limiting colour standards, etc.), the test has great adaptability, as has been strikingly demonstrated by its application to milk for which pasteurizing requirements vary from country to country. It can therefore be confidently expected that further research will achieve for milk products what it has already achieved for milk itself, namely, the provision in the phosphatase test of a safe and reliable method for controlling and supervising pasteurization.

APPENDIX.

METHODS FOR PERFORMING THE PHOSPHATASE TEST.

Precautions.

(1) As the test is based on the estimation of minute quantities of phenol, care should be taken to keep phenols, disinfectants containing phenols and soap containing carbolic acid at a safe distance from the test reagents and apparatus used.

(2) The use of bottle caps made from phenolic resins should be avoided.

(3) Stoppers may contain phenolic impurities and fresh batches should be tested before use.

(4) All glassware should be carefully cleaned and rinsed thoroughly before use. The use of disinfectant soap should be strictly avoided.

(5) Contamination by saliva, which is known to contain considerable amounts of phosphatase, should be avoided.

(6) All reagents should be kept in a dark, cool place, and well protected from dust. Their purity should be tested at frequent intervals (see " blank " tests).

(7) In taking samples of milk the usual precautions should be observed. No preservatives should be added, except when Scharer's technique is used, in which case the addition of 0·8g. of borax to 100 ml. of milk is permissible.

(8) Where possible, the phosphatase test should be carried out within 18 hours of pasteurization.

(9) When it is necessary to delay testing, samples should be kept in cold store to prevent bacterial growth. Refrigerated samples should be warmed up before testing.

(10) Samples which show a definite taint and/or clot on boiling should not be admitted for testing.

(11) A fresh pipette should be used for each test in order to avoid contamination by raw or underpasteurized milk.

(12) Tests should not be carried out in direct sunlight.

(13) " Control " tests should be made whenever a positive phosphatase test is obtained.

A.—TECHNIQUE OF THE KAY-GRAHAM TEST (27, 44, 45, 81).

(a) Reagents.

(i) *Buffer substrate :* Dissolve 1·09 g. of disodium phenyl phosphate and 11·54 g. of " sodium veronal " (sodium diethyl barbiturate) in water saturated with chloroform and make up to 1 litre. Add a few drops of chloroform to prevent the growth of micro-organisms, and keep in the refrigerator. Stable for months unless contaminated with certain moulds.

Alternatively, use buffer substrate tablets.* One tablet should be dissolved in 50 ml. of distilled water saturated with pure chloroform. (If this solution is not made up fresh on each occasion, then it should be kept in a refrigerator with an extra drop or two of chloroform).

(ii) *Folin and Ciocalteu's phenol reagent** : Dissolve 100 g. of sodium tungstate, $Na_2WO_4.2H_2O$, and 25 g. of sodium molybdate, $Na_2MoO_4.2H_2O$, in 700 ml. of water in a 1500 ml. flask connected, preferably by ground glass joint, with a reflux condenser. Add 50 ml. of syrupy (85 per cent.) phosphoric acid and 100 ml. of concentrated hydrochloric acid. Reflux the mixture gently for 10 hours. (If an all-glass connection between flask and condenser is not available, use a rubber stopper or cork wrapped

* Obtainable from The British Drug Houses, Ltd.

in tin foil. Take the greatest care that the solution does not come in contact with the tin foil.) After 10 hours cool, add 150 g. of pure lithium sulphate, 50 ml. of water and a few drops (usually 4–6) of liquid bromine. Boil the mixture under the hood without the condenser for 15 minutes to boil off excess of bromine. Cool, dilute to 1 litre and filter. The finished reagent should have a golden yellow colour with no greenish tint. Any reagent with a greenish tint should be rejected. Keep in a refrigerator and protected from contact with dust, metal, etc. and any reducing substance. Stable for at least four months. The reagent is diluted with twice its volume of distilled water before use.

(iii) 14 per cent. solution (weight per volume) of pure anhydrous *sodium carbonate*. It is advisable to standardize this solution by titration.

(b) Apparatus.

(i) For most purposes a simple comparator* with one or more standard coloured Lovibond glasses provides the necessary measuring instrument. (The colour produced under the conditions of the test is not a true blue, but contains some red and yellow. These are provided in the standard coloured glasses.)

If a Lovibond tintometer* is available the colour produced in the test may be accurately measured. For this purpose it is important that a 13 mm. cell should be used in order that the readings may be correlated with those of Kay and Graham.

(ii) A water bath which can be kept at 47(116·6)±2(3·6)° for a short time (10 minutes).

(iii) A water bath or incubator maintained at 37(98·6)—38(100·4)°.

(iv) Whatman filter papers, 9 cm. No. 30 or No. 40 (preferably the latter).

(v) A number of 0·5 ml. pipettes, grade A or N.P.L. are recommended. A separate pipette is required for each milk sample.

In addition to the above a small number of burettes for measurement of certain of the solutions used, a good supply of fairly stout test tubes, $\frac{5}{8}$ inch diameter, marked at 10 ml., with rubber stoppers to fit, and a number of filter funnels to take the 9 cm. filter papers, are required.

Test A. (Short Test).

This test can be carried out in $\frac{1}{2}$ hour, and is useful for determining whether a milk has been heated or not, and for observing gross errors of pasteurization technique.

(*a*) *Determination.*

To 10 ml. of the buffer substrate solution contained in a 25 ml. test tube, add 0·5 ml. of the well mixed milk, mix thoroughly, and incubate the mixture in a water bath at 47(116·6)±2(3·6)° for 10 minutes. Remove the tube and cool to 15(59)—20(68)° by immersion in cold water. Add 4·5 ml. of the diluted Folin-Ciocalteu reagent, allow to stand for 3 minutes, and filter. To 10 ml. of the filtrate, taken immediately after filtration, add 2 ml. of the sodium carbonate solution, mix and place the tube in boiling water (kept boiling) for 5 minutes and again filter.

The test should be carried out in duplicate, and at the same time duplicate control tests should be made in the following manner:

(*b*) *Control tests.*

Mix thoroughly 10 ml. of the buffer substrate solution with 4·5 ml. of the diluted Folin-Ciocalteu reagent and 0·5 ml. of the milk, allow to stand for 3 minutes, and filter. To 10 ml. of the filtrate add 2 ml. of the sodium carbonate solution, mix and place the tube in boiling water (kept boiling) for 5 minutes, and again filter.

Compare the colours of the four tubes with those of the standard glasses.

*Made by Tintometer Ltd., Salisbury, England.

(*c*) *Interpretation.*

If the controls show more than a trace of blue colour and the reagents contain no free phenol, it is probable that a phenol-producing organism is present in the milk. This does *not* occur in pasteurized milk which has been kept at a satisfactorily low temperature following pasteurization. With fresh, properly pasteurized milk, that is, milk which has been cooled to 12·8(55)° immediately after pasteurization and maintained between that temperature and 18·3(65)° for not more than 18 hours, the control tubes should show only a trace of blue colour. If with such controls the *incubated* tubes show a definite blue colour, *i.e.* over 2·3 units, it can be said with certainty that the milk has not been adequately pasteurized, and if the colour is over 6·0 blue units, the milk has very probably not been heated at all. With some experience, it will be possible to omit the control tubes for the majority of samples submitted to this test.

While the production of a colour exceeding the standard may be taken as evidence of imperfect pasteurization or admixture of raw milk, the converse is not invariably true. For example, if the milk has been heated at 60(140)° instead of at (or above) the statutory minimum temperature of 62·8(145)° the colour produced in test A will not exceed the standard. In cases where the colour approaches very near to the standard tint, or where more accurate information is required regarding the efficiency of pasteurization, and generally, when time permits, the following more delicate Test B should be applied.

Test B. (Long Test).

This test detects small as well as gross errors in processing, and is the one most generally used in the control laboratory.

(*a*) *Determination.*

To 10 ml. of the buffer substrate solution contained in a 25 ml. stoppered test tube, add 0·5 ml. of the well mixed milk, and mix thoroughly. Add 3 drops of chloroform, stopper the tube, and incubate at 37(98·6)–38(100·4)° for 24±2 hours. At the end of this time, cool, add 4·5 ml. of the diluted Folin-Ciocalteu reagent, mix, allow to stand for 3 minutes, and filter. To 10 ml. of the filtrate add 2 ml. of the sodium carbonate solution, mix and place the tube in boiling water (kept boiling) for 5 minutes and again filter. Compare the colour in the tintometer or comparator.

(*b*) *Control tests.*

Tests should always be made in duplicate, and control tests carried out when necessary. The controls in Test B should either be completed within a few minutes as in Test A, or should be filtered at the stage reached after adding the Folin-Ciocalteu reagent and be kept in the refrigerator until the next day. In practice, a useful alternative is to keep the milk samples in the refrigerator for 24 hours after the two experimental tubes have been put into the incubator and only carry out controls the following day on those samples which show a positive test after 24 hours' incubation. The colour of the filtrate from control tests should not exceed 1·5 blue units.

(*c*) "*Blank*" *test.*

To test the purity of the reagents a "blank" test should be carried out by incubating a tube containing buffer substrate and chloroform, but *no* milk with each batch of samples. Such a blank should give a colour reading of not more than 0·5 Lovibond blue units.

(*d*) *Interpretation.*

Milks which, with controls below 1·5 units, give colours below 2·3 blue units may be classified as properly pasteurized. Those giving colours between 2·3 and 6·0

blue units should be classified as improperly pasteurized, while those giving colours greater than 6·0 units show evidence of serious errors in pasteurization performance. Raw milk usually gives more than 30 units of colour.

It is not possible by the phosphatase test alone to decide what is the specific cause of a high figure. All that can be said is that the milk has not been correctly pasteurized. Test B is capable of detecting with certainty the following faults :

(*a*) Holding at 62·8(145)° for 20 instead of 30 minutes.

(*b*) Holding at 0·85(1·5)° below the minimum temperature (*i.e.* holding at 61·9 (143·5)° or below).

(*c*) Admixture of 0·25 per cent. of raw milk with correctly pasteurized milk.

B.—NEAVE'S MODIFICATION (45).

Proceed as under A except for the following alterations.

(a) Reagents.

Sodium hexametaphosphate. Use the salt in the form of flakes, and sift in order to remove any white powder which has formed on storage. Always keep in a tightly stoppered bottle. Prepare a 5 per cent. solution by dissolving the salt in warm water and making up after cooling.

To 2 volumes of this solution add 1 volume of the (undiluted) stock solution of Folin and Ciocalteu's reagent. The mixture is stable for several weeks.

(b) Technique.

Use 4·5 ml. of the above mixture instead of 4·5 ml. of diluted Folin's reagent. After addition of 2·0 ml. of 14 per cent. sodium carbonate solution place in boiling water for only 2 instead of 5 minutes. Cool and proceed to read the colour developed on boiling. (The presence of hexametaphosphate prevents the formation of a precipitate, so that the second filtration process can be dispensed with).

C.—GILCREAS AND DAVIS' MODIFICATION (12, 41, 81).

(*Tentative Method—Association of Official Agricultural Chemists*).

(a) Reagents.

As under A.

(b) Apparatus.

Test tubes, 20 mm. × 160 mm.
Test tubes, 13 mm. inside diameter.
Paper towelling.
Water-bath.
Incubator or bath at 34(93·2)–37(98·6)°.
Funnels to take 11 cm. filter paper.
Filter paper, Whatman, No. 40, 11 cm. diameter. (Use only this grade as interfering substances are at times extracted from other grades).
Daylight lamp.
Turquoise blue, unglazed, opaque glass plate.

(c) Permanent Phenol Standards.

(i) Colour solution, grey. Dissolve in distilled H_2O 31·9 g. of cobalt chloride, $CoCl_2.6H_2O$, 67·5 g. of copper sulphate, $CuSO_4.5H_2O$, 75 g. of nickel sulphate, $NiSO_4.6H_2O$, add 32 c.c. of HCl conc. and 45 c.c. of H_2SO_4 conc., and dilute to 500 c.c.

(ii) Colour solution, red. Dissolve 476 g. of cobalt chloride, $CoCl_2.6H_2O$, in distilled H_2O, and filter. To filtrate add 100 c.c. of HCl conc. and dilute to 1 litre.

(iii) Colour solution, blue. Dissolve 300 g. of copper sulphate, $CuSO_4.5H_2O$, in distilled H_2O, add 20 c.c. of H_2SO_4 conc., and dilute to 1 litre. (Should crystals appear when solution is cooled to below 20(68)° warm slightly before using to insure complete solubility).

TABLE I.

Preparation of permanent phenol standards.

Phenol mg./0·5 c.c.	Colour solution.		
	Grey (i) c.c.	Red (ii) c.c.	Blue (iii) c.c.
0·01	0·30	0·106	0·96
0·02	0·40	0·140	1·16
0·03	0·55	0·180	1·65
0·04	0·65	0·216	2·10
0·06	0·92	0·286	3·00
0·09	1·30	0·326	4·40
0·12	1·70	0·360	5·70
0·15	2·50	0·396	7·10

Prepare permanent colour standards equivalent to phenol concentrations of from 0·01 to 0·15 mg. per 0·5 c.c. of sample by combining the quantities of colour solutions (i), (ii), (iii) indicated in Table I, and diluting to 10 c.c. with distilled H_2O in each case ; *e.g.* 0·3 c.c. of solution (i) +0·106 c.c. of solution (ii) +0·96 c.c. of solution (iii), + H_2O to make a vol. of 10 c.c., which is equivalent to a phenol concentration of 0·01 mg. in 0·5 c.c. of sample.

These colour standards are suitable for use only in natural daylight. If, however, a turquoise blue, unglazed, opaque glass plate is used to deflect the light from a daylight lamp through the tubes of standards and sample, accurate colour comparisons can be made in the absence of daylight. Since the standards are prepared for use only at 13 mm. depth of colour, tubes of different diameters cannot be used for accurate work.

(d) DETERMINATION.

Transfer 10 c.c. of the buffer substrate solution into a test tube 20 mm. × 160mm. and add 0·5 c.c. of the milk to be tested. Add a few drops of $CHCl_3$, mix thoroughly by rotating the tube, and cover to protect contents from dust. (Do not use rubber or cork stoppers ; paper towelling placed over the open end of the tubes is satisfactory). Warm to 37(98·6)–39(102·2)° in a water bath and incubate at 34(93·2)–37(98·6)° for not less than 18 hours and not more than 24 hours. After incubation add 4·5 c.c. of the diluted Folin-Ciocalteu reagent. Mix and allow to stand for 3 minutes. Filter and transfer 5 c.c. of the filtrate to a test tube. Add 1 c.c. of the 14 per cent. anhydrous Na_2CO_3 solution and mix thoroughly by rotating the tube. Place the tube in a boiling water bath for 5 minutes and filter into a test tube of 13 mm. diameter. Cool and estimate the colour of the filtrate by comparison with the permanent colour standards.

(e) CONTROL TEST.

(To check deterioration of reagents and the presence of interfering substances in the milk sample).

To 10 c.c. of the buffer substrate solution add 4·5 c.c. of the diluted Folin-Ciocalteu reagent and 0·5 c.c. of the milk sample. (Do not incubate). Mix thoroughly, allow to stand for 3 minutes and filter. To 5 c.c. of the filtrate add 1 c.c. of the 14 per cent. Na_2CO_3 solution, mix thoroughly by rotating the tube, heat in a boiling water bath for 5 minutes and filter into a test tube of 13 mm. diameter. Cool and compare the colour of the filtrate with the permanent colour standards. If the phenol value obtained is greater than 0·02 mg. subtract the excess from the phenol value of the incubated sample to obtain value indicative of pasteurization treatment.

(f) Interpretation.

A phenol value of 0·04 mg. of phenol per 0·5 c.c. of sample generally indicates milk heated to 61·7(143)° for 30 minutes. A value greater than this indicates progressively inadequate heat treatment. In reporting results, give the mg. of phenol per 0·5 c.c. of sample as well as an interpretation as to whether the milk is pasteurized or underpasteurized.

As a result of further experience Gilcreas (42) now recommends interpretation on the following basis : a phenol value of less than 0·05 mg. per 0·5 ml. as indicative of proper pasteurization, a value of 0·05–0·08 mg. per 0·5 ml. as indicative of slight underpasteurization, a value between 0·09 and 0·15 mg. per 0·5 ml. as indicative of underpasteurization and a value of 0·15 mg. per 0·5 ml. or greater as indicative of gross underpasteurization.

D.—SCHARER'S MODIFICATION (1, 50, 51, 52, 81).

1. Laboratory Test.

(a) Reagents.

(i) *Borate buffer.* Dissolve 28·427 g. of sodium borate ($Na_2B_4O_7.10H_2O$) in 900 c.c. of water. Add 3·27 g. of NaOH (81·75 c.c. N-NaOH solution) and dilute to 1 litre.

(ii) *Gibbs' phenol reagent.* Dissolve 40 mg. of 2 : 6-dibromoquinone chloroimide (referred to below as BQC) in 10 c.c. of methyl or 95 per cent. ethyl alcohol. Keep reagent tightly stoppered and under refrigeration.

(iii) *Lead acetate solution.* Dissolve 50 g. of lead acetate ($Pb(C_2H_3O_2)_2.3H_2O$) in 100 c.c. of water.

(iv) *Buffer substrate.* Dissolve 0·5 g. of *crystalline* disodium phenyl phosphate in 5 c.c. of water in a small (10×100 mm.) test tube. Add 0·5 c.c. of borate buffer. Shake well and add 1/25 c.c. of BQC solution. (Or add two drops from the dropper delivering 50 drops per c.c. of the BQC solution). Shake well. Allow 5 minutes for colour development. Extract indophenol by shaking with 2 c.c. of neutral *n*-butyl alcohol. Allow to stand until alcohol has separated completely. Remove supernatant alcohol layer with a pipette or medicine dropper and discard. Dilute remainder with 100 c.c. of borate buffer and sufficient water to make 1 litre. This buffer substrate is phenol-free. Store under refrigeration. Because of possible decomposition, prepare quantities of this reagent sufficient for immediate needs only. The pH of this solution is approximately 9·6 (blue to thymolphthalein solution—0·04 per cent. in 50 per cent. ethyl alcohol).

Tablets are available for convenient preparation of small quantities of the buffer substrate and BQC solutions.*

(v) *Sodium pyrophosphate solution :* 10 g. of $Na_4P_2O_7.10H_2O$ in 100 c.c. of water.

(vi) *n-Butyl alcohol :* see Field Test.

* Obtainable from R. P. Cargylle, 118, Liberty St., New York, N.Y.

(b) Apparatus.

Pipette, 1 c.c.
Test tube, pyrex, 15 mm. × 125 mm.
Test tube, 6 × $\frac{5}{8}$ inch.
Incubator or waterbath set at 37(98·6)–45(113)°.
Water bath.
Funnel.
Filter paper (any inexpensive qualitative paper).
Dropper.
Double fluxed glass or sheet of Plastacele No. C-1605 HH, 0·015 in. thick.
Standard colour solutions.

(c) Permanent Colour Standards.

Laboratory test—acid series.

(i) Colour solution, red. 0·5N cobalt chloride in 1 per cent. HCl (59·59 g. of $CoCl_2.6H_2O$ per litre).

(ii) Colour solution, blue. 30 per cent. copper sulphate in 1 per cent.H Cl (300 g. of $CuSO_4.5H_2O$ per litre).

(iii) Colour solution, yellow. 0·5N ($\frac{M}{6}$) ferric chloride in 1 per cent. HCl (45·05 g. of $FeCl_3.6H_2O$ per litre).

Combine quantities indicated in table following and dilute to 5 c.c. with water.

Colour Solutions.

Units.	Blue c.c.	Red c.c.	Yellow c.c.
1	0·2	0·35	0·5
2	0·5	0·6	0·55
3·5	0·5	0·5	0·5
5	1·0	0·75	0·5
7·5	1·5	0·75	0·5
10	2·0	1·0	0·25

The following ammoniacal colour standards supplement the acid series above by affording a more extensive range.

Laboratory test—ammoniacal series.

(i) Colour solution, red. Dissolve 1·8 g. of roseo (aquopentamine) cobaltic chloride, $(Co(NH_3)_5.H_2O)Cl_3$, per litre of 2·8 per cent. ammonium hydroxide.

(ii) Colour solution, yellow. Dissolve 0·84 g. of ammonium chromate, $(NH_4)_2CrO_4$, per litre of 2·8 per cent. ammonium hydroxide.

(iii) Colour solution, blue. Dissolve 6·24 g. of copper sulphate $CuSO_4.5H_2O$ per litre of 2·8 per cent. ammonium hydroxide.

Combine quantities indicated in table following and dilute to 5 c.c. with water.

Colour Solutions.

Units	Blue c.c.	Yellow c.c.	Red c.c.
1	0·25	0·4	0·5
2	0·5	0·5	0·75
3·5	0·8	0·7	1·0
5	1·0	0·55	0·6
7·5	1·25	0·75	0·75
10	1·5	0·5	0·25
15	2·5	0·5	
20	2·75	0·5	
25	3·0	0·5	
50	3·75	0·5	
100·	4·0	0·5	
500	4·5	0·5	

These colour standards are suitable for use in natural or artificial light provided an opal double fluxed glass or sheet of Plastacele No. C-1605 HH, 0·015 inch thick is used as a light filter. Test tubes similar to those in which each test is conducted should be used for colour standards.*

(d) Determination.

Transfer 1 c.c. of the milk to be tested to a pyrex test tube (15 mm. × 125 mm.) and add 10 c.c. of the buffer substrate. Mix thoroughly. Warm to about 40(104)° and place in an incubator or water-bath for 1 hour at 37(98·6)–45(113)° [41(105·8)° preferred]. After incubation, place tube in boiling water for 5 minutes. Cool in ice water. Add 0·1 c.c. of lead acetate solution. Shake immediately and well. Proteins will coagulate and separate sharply. In some instances it may be necessary to add an additional 0·05 c.c. of lead acetate solution. Filter. To 5 c.c. of filtrate in a test tube (6 × $\frac{5}{8}$ inches), add 0·5 c.c. of borate buffer. Addition of a few drops of the pyrophosphate solution will clarify filtrate turbidity if any. Add 0·04 c.c. of BQC solution (2 drops from recommended dropper). Mix thoroughly by rotating tube. After 15 minutes, estimate colour by comparison with permanent colour standards.

(e) Control Tests.

To check presence of interfering substances in milk sample. To 9 c.c. of water add 1 c.c. of borate buffer and 1 c.c. of milk sample. Mix well, place in boiling water for 5 minutes, and follow through as in determination (d). Development of blue colour indicates extent of indophenol colour due to interfering substances in milk sample.

* Another method of making a colour standard is to employ a 0·002 per cent. solution of sodium sulfocarbolate. 5 c.c. of this solution when treated with the buffering agent and BQC solution is equivalent to 2 units. This solution should be prepared weekly (51, 52).

To check reagents. To 5 c.c. of buffer substrate, add 1/25 c.c. of BQC solution. Development of blue colour in 15 minutes indicates extent of substrate decomposition. Or, incubate 10 c.c. of buffer substrate with 1 c.c. of a boiled milk sample. Proceed as under determination (d). Indophenol development indicates extent of substrate decomposition. Avoid intimate contact of BQC solution with rubber. A darkening of this solution indicates decomposition.

(f) Interpretation.

A phenol value of 2 units or less generally indicates milk heated to 61·7(143)° for 30 minutes. Commercially pasteurized milk usually yields 0 to 1 unit because of added time exposure incurred by operating variations in preheating, filling and emptying of tanks, etc. A value of 2 units or greater in commercially pasteurized milk indicates inadequate heat treatment.

2. Field Test.

Solutions of buffer substrate and BQC whether prepared from reagents or tablets (see above) may be used in the field test.

(a) Reagents.

(i) *Neutral n-butyl alcohol* (boiling range 115(239)–118(244·5)°). The *n*-butyl alcohol should be colourless, of good quality and free from excessive acids or esters. If the alcohol utilized is very old, it should be redistilled with sodium hydroxide. To test the suitability of the alcohol, shake a sample with an equal quantity of neutral distilled water, allow it to separate and test the water layer with brom thymol blue. A blue or green colour should result.

(ii) *BQC solution*—as indicated above. If tablets are used, one tablet is equivalent to 20 mg. of BQC and should be dissolved in 5 c.c. of ethyl or methyl alcohol.

(iii) *Buffer substrate solution*—as indicated above. If tablets are used: Crush buffered substrate tablet in test tube, dissolve in 5 c.c. of distilled water. Add 2 drops of BQC solution. Allow 5 minutes for colour development, then extract the indophenol with 2 to 2·5 c.c. of *n*-butyl alcohol. Allow to stand until alcohol layer has separated at top of tube. Remove alcohol layer with medicine dropper and discard. Dilute remainder of solution to 50 c.c. This solution is then phenol free.

Both solutions decompose with age and should be stored under refrigeration or prepared shortly before use.

(b) Apparatus.

Test tubes 10 × 110 mm. calibrated at 5 c.c., 5·5 c.c. and 7·5 c.c. measured to top of meniscus. *Gum* rubber stoppers to fit.

Medicine droppers for use as pipettes.

Small ($\frac{1}{4}$ oz.) brown glass pharmaceutical dropping bottle with dropper calibrated to deliver 50 drops per c.c. of BQC solution.

(c) Permanent Colour Standards.

(i) Colour solution, red. Same as acid series above.
(ii) Colour solution, yellow. Same as acid series above.
(iii) Colour solution, blue. 0·5N copper sulphate in 1 per cent. HCl (62·425 g. of $CuSO_4.5H_2O$ per litre).

Combine quantities indicated in table following, and dilute to 5 c.c. with water.

Colour Solutions.

Units	Red c.c.	Blue c.c.	Yellow c.c.
2	0·4	1·5	0·5
5	0·2	2·2	0·5

(d) Determination.

To 5 c.c. of buffered substrate, add 0·5 c.c. of milk sample. Shake briefly. Incubate for 10 minutes in a water-bath at 36(96·8)–44(111·2)°. If no water-bath be available, incubate in pocket for a 15 to 20 minute period. Discontinue incubation, add 6 drops of BQC solution. Shake well immediately. Allow to stand for 5 minutes. Appearance of blue colour indicates inadequate pasteurization. For greater sensitivity add 2 c.c. of neutral *n*-butyl alcohol. Extract indophenol by completely inverting tube ten times, pausing after each 180° arc to allow bubbles to break and alcohol to separate. Rapid inversion will result in an emulsion, but if correctly performed a clear supernatant alcohol layer $\frac{3}{4}$ inch in height will result. Compare with colour standards for field test using recommended filter.

A reagent blank should be made by adding 3 drops of BQC solution to 5 c.c. of the substrate. If a blue colour results, the substrates solution should be discarded. If the butyl alcohol procedure is utilized this reagent blank should be extracted with the alcohol.

(e) Interpretation.

A value equal to or greater than 2 units generally indicates faulty pasteurization.

E.—LEAHY'S MODIFICATION (57, 58, 81).

(a) Reagents.

(i) *Buffer substrate.* Dissolve 1·09 g. of disodium phenyl phosphate and 11·54 g. of sodium veronal (sodium diethyl barbiturate) in water and dilute to 1 litre. Add from 2 to 5 c.c. of $CHCl_3$ per litre, and store in the refrigerator.

(ii) *Gibbs' phenol reagent.* 2 : 6-dibromoquinone chloroimide solution. Dissolve 0·2 g. of the pure salt in 50 c.c. of 95 per cent. C_2H_5OH, place in a glass stoppered bottle, and store in the refrigerator. The solution should be discarded after storage for 1 week.

(iii) *Standard phenol solution.* Dissolve slightly more than 1 g. of pure phenol in water, and standardize by the procedure outlined in the U.S. Pharmacopœia XI, p. 285, as follows : Dissolve about 1·5 g. of phenol, accurately weighed, in sufficient distilled water to make 1,000 c.c. of solution. Transfer an aliquot part of the solution, containing from 0·038 to 0·041 g. of phenol, to a 500 c.c. glass stoppered flask having a long narrow neck, add 30 c.c. of tenth-normal bromine, then 5 c.c. of hydrochloric acid, and immediately insert the stopper. Shake the flask repeatedly during $\frac{1}{2}$ hour, allow it to stand for 15 minutes, remove the stopper just sufficiently to introduce quickly 5 c.c. of an aqueous solution of potassium iodide (1 in 5), being careful that no bromine vapor escapes, and at once stopper the flask. Shake the latter thoroughly, remove the stopper, and rinse it and the neck of the flask with a little distilled water, so that the washings may flow into the flask, then add 1 c.c. of chloroform, shake the mixture well, and titrate the liberated iodine with tenth-normal sodium thiosulphate,

using starch T.S. as the indicator. Each c.c. of tenth-normal bromine is equivalent to 0·001568 g. of C_6H_5OH. Dilute the required amount of this solution to 100 c.c. to give a solution containing 0·10 mg. of phenol per c.c.

(iv) *Chloroform.*

(b) Apparatus.

Test tubes.
Pipettes, 1 c.c. and 0·1 c.c.
Incubator or water bath set at 37(98·6)°.

(c) Procedure.

Add 1 c.c. of sample of milk to 10 c.c. of the buffer substrate solution add a drop of $CHCl_3$, mix thoroughly by rotating the tube, and incubate at 37(98·6)° for not less than 12 and not more than 18 hours. After incubation, add 0·1 c.c. of the 2 : 6-dibromoquinone chloroimide solution and mix immediately. Compare the colour with colours obtained in the controls of boiled milk, and boiled milk containing 0·01 mg. of phenol per c.c.

(d) Interpretation.

The absence of a blue colour indicates the sample was heated to 61·7(143)° for 30 minutes. A colour of the same or greater intensity than that of the 0·01 mg. of phenol control indicates inadequate pasteurization.

F.—ASCHAFFENBURG AND NEAVE'S MODIFICATION (55).

(a) Reagents.

(i) *Buffer :* 0·5N sodium carbonate (dissolve 2·65 g. of pure anhydrous Na_2CO_3 in 100 ml. of distilled water). Stable for at least 2 weeks, if kept stoppered.

(ii) *Buffer-substrate :* dissolve 0·11 g. of disodium phenyl phosphate in 80–90 ml. of distilled water, add 3 ml. of buffer and make up to 100 ml. This solution should be used within a few hours of preparation. Fresh supplies of disodium phenyl phosphate should be tested before acceptance (see "blank" test). No chloroform should be added.

(iii) *Gibbs' phenol reagent :* dissolve 23 ± 0·5 mg. of 2 : 6-dibromoquinone chloroimide (BQC) in 5 ml. of absolute alcohol. Keep in a good quality *brown* bottle. The solution is stable for a week at room temperature and for a month when kept at a temperature below 7(44·6)°. It should be discarded as soon as it shows signs of becoming brown or reddish brown. Care should be taken to use only pure, canary yellow BQC powder in preparing the solution.

(iv) *Normal butyl alcohol :* neutralize a good quality alcohol by adding NaOH using thymol blue or other convenient indicator.

(b) Apparatus.

(i) *Pipettes :* a few 5 ml. pipettes and a number of 1 ml. and 10 ml. graduated pipettes.

(ii) *Waterbath :* heated to 37(98·6)–38(100·4)°.

(iii) *Test tubes :* Length 150 mm., internal diameter 13·5 ± 0·5 mm.

It is important to use test tubes of the above dimensions as their size is not without influence on the results of the test.

(iv) *Rubber stoppers :* to fit test tubes. They should be kept in water and boiled out before use.

(v) *Glass dropper :* for BQC solution.

This should be tested to deliver 50 ± 2 drops of BQC solution per ml. Use in vertical position.

(c) Determination.

1. Transfer 5 ml. of freshly prepared buffer-substrate solution to a test tube and add 0·5 ml. of the well mixed milk.
2. Stopper, shake 8–10 times and incubate for 1 hour at 37(98·6)–38(100·4)°. Incubate, at the same time, a " blank " *i.e.* 5 ml. of buffer-substrate solution to which no milk has been added.
3. After 1 hour, take the tubes from the bath, remove stoppers and start at once to add 5 drops of BQC solution. Add to one or two tubes at a time and mix immediately, using about ten circular motions. (If the contents of the test tubes are allowed to cool below 30(86)° before addition of the BQC solution, variations in the strength and shade of indophenol colour will occur. To avoid such variations it is also essential to use exactly 5 drops of BQC solution). After all the tubes have been thus treated, replace stoppers and shake about 10 times. Use only 1 drop of BQC solution for the " blank " test.
4. Remove stoppers, add 2 ml. of butyl alcohol and replace stoppers.
5. Cool the tubes to 15(59)–20(68)°. Commence to extract the indophenol colour 5–10 minutes after the addition of the BQC solution. Working with pairs, invert the tubes slowly 8 times (4 complete turns). Replace tubes in a rack after each inversion and do not invert again until the two layers have clearly separated. The tubes should be tilted slowly until the liquid just reaches the opposite end and then brought to the vertical position a little more quickly in such a manner that about ½ minute is required for the two layers to separate clearly.* The colour extraction should be completed within 20 minutes after removal of the tubes from the water bath.
6. Read the colour developed in the butyl alcohol layer (see below).

(d) " Control " Tests.

In addition to " blank " tests which should be carried out with each batch of tests, it is necessary to perform control tests in all cases in which it is suggested that the colour development in the test proper might be due to causes other than milk phosphatase. Add 0·5 ml. of the milk to 5 ml. of a solution containing 3 ml. of the carbonate buffer, but no substrate, in 100 ml. of water, incubate for 1 hour at 37(98·6)–38(100·4)° or just warm to this temperature and then proceed as usual.

(e) Methods for Colour Reading.

(i) *By means of the Lovibond tintometer.* For accurate readings of the indophenol colour the Lovibond tintometer may be used. The butyl alcohol layer is syphoned off and transferred to a ½ inch-square cell (as used in the Hellige comparator), but as 2 ml. are insufficient to give the depth of liquid required to cover the opening of the tintometer, it is necessary to perform the phosphatase test in duplicate and to combine the butyl alcohol layers. The indophenol colour is matched by the use of blue, yellow and " neutral " glasses.

(ii) *By means of inorganic colour standards.* For routine testing the quicker method of comparing the colour of the butyl alcohol layer with that of one or more colour standards is more satisfactory. Following Scharer's recommendation three stock

* With a little practice it is quite easy to obtain maximum extraction of the indophenol colour and to avoid delay in the separation of the layers caused by too rapid inversion of the tubes.

solutions are made up for the preparation of the colour standards as under D (p. 48). The actual colour standards are prepared by mixing these solutions and water in the following proportions :

	Blue	Yellow	Red	H_2O
I	4·6	3·0	0·9	1·5
II	5·5	2·7	0·8	1·0

They should be kept in the dark and checked at frequent intervals. They are stable for at least one month. For comparison of the colour extracted in the test with the standards, it is desirable to examine the test tubes in reflected light, holding them close to a white background, and to interchange their positions. No attempt should be made to match the colours, but rather to determine whether the test solutions contain more or less of the blue component than the standards.

(f) Interpretation of Results.

(i) Before extraction with butyl alcohol. A blue or blue grey colour is indicative of gross errors in pasteurizing technique or of serious contamination with raw milk.

(ii) After extraction with butyl alcohol. Classify in accordance with the following table :

Colour of butyl alcohol layer	Lovibond blue units	Blue compared with colour standards	To be classified as :
Yellow or greenish yellow	<2·0	Less than standard I	Properly pasteurized
Yellowish green	2·0–2·5	Between standards I & II	" Doubtful "
Green or bluish green	>2·5	More than Standard II	Under-pasteurized

" *Blank* " *tests :* No greenish or bluish colour should develop either before or after extraction. In case of colour development test buffer and substrate separately.

" *Control* " *tests :* Normal milk should give a colourless or yellowish butyl alcohol layer. A green or blue colour indicates presence of phenol.

(g) A Quick Test for Raw Milk.

A quick test to distinguish raw from heated milk can be obtained by adding 5 drops of BQC solution to a mixture of 5 ml. of buffer substrate and 0·5 ml. of milk at room temperature (15(59)–20(68)°). In the presence of raw milk a bluish colour develops within less than 3 minutes.

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE
LIBRARY
MYSORE

REFERENCES.

1. Scharer (1938), J. Dairy Sci., **21**, 21.
2. Orla-Jensen (1932), Z. Untersuch. Lebensmitt., **63**, 300.
3. Burstein & Frum (1931), Z. Untersuch. Lebensmitt., **62**, 489.
4. Lane-Claypon (1916), Milk and its hygienic relations. London: Longmans, Green & Co.
5. Smidt (1908), Arch. Hyg., **58**, 313.
6. Schardinger (1902), Z. Untersuch. Nahr.- u. Genussm., **5**, 1113.
7. Bengen & Bohm (1934), Z. Untersuch. Lebensmitt., **67**, 379.
8. Kluge (1933), Z. Untersuch. Lebensmitt., **65**, 71.
9. Rothenfusser (1930), Z. Untersuch. Lebensmitt., **60**, 94.
10. Gould (1932), J. Dairy Sci., **15**, 230.
11. Richardson & Hankinson (1936), J. Dairy Sci., **19**, 761.
12. Gilcreas & Davis (1936), Proc. int. Ass. Milk Sanit., 15.
13. Storch (1899), Z. Untersuch. Nahr.- u. Genussm., **2**, 239.
14. Bouma & van Dam (1918), Biochem. Z., **92**, 385.
15. Kay & Graham (1933), J. Dairy Res., **5**, 63.
16. Folley & Kay (1937), Tabul. biol., Berl., **6**, 268.
17. Folley & Kay (1936), Ergebn. Enzymforsch., **5**, 159.
18. Graham & Kay (1933), J. Dairy Res., **5**, 54.
19. Demuth (1925), Biochem. Z., **159**, 415.
20. Wilson & Hart (1932), J. Dairy Sci., **15**, 116.
21. Folley & Kay (1936), Enzymologia, **1**, 48.
22. Folley & White (1936), Proc. roy. Soc., B., **120**, 346.
23. Kay (1925), Biochem. J., **19**, 433.
24. Folley & Kay (1935), Biochem. J., **29**, 1837.
25. Koppejan (1936), Chem. Weekbl., **33**, 747.
26. Briggs (1922), J. biol. Chem., **53**, 13.
27. Kay & Graham (1935), J. Dairy Res., **6**, 191.
28. Stein (1937), Disputats, Kobenhavn.
29. Jepsen & Madelung (1939), Medlemsbl. danske Dyrlaegeforen., **22**, Nos. 4 & 5.
30. King & Armstrong (1934), Canad. med. Ass. J., **31**, 376.
31. Folin & Ciocalteu (1927), J. biol. Chem., **73**, 627.
32. Michaelis (1930), J. biol. Chem., **87**, 33.
33. Krueger (1938), Amer. J. publ. Hlth., **28**, 320.
34. Krueger (1938), J. Milk Tech., **1** (2), 36.
35. Smith (1938), Canad. publ. Hlth J., **29**, 288.
36. Geiger & Davis (1937), J. Amer. med. Ass., **109**, 1363.
37. Spencer (1938), Canad. Dairy Ice Cr. J., **17** (6), 21.
38. Storrs & Burgwald (1938), J. Milk Tech., **1** (2), 18.
39. Burgwald & Giberson [Gilbertson] (1938), J. Milk Tech., **1** (7), 11.
40. Gilcreas (1938), J. Ass. off. agric. Chem., Wash., **21**, 372.
41. (1938), J. Ass. off. agric. Chem., Wash., **21**, 82.
42. Gilcreas (1939), Amer. J. publ. Hlth., **29**, 158.
43. Anderson, Herschdörfer & Neave (1937), Analyst, **62**, 86.
44. Kay & Neave (1937), Dairy Industr., **2**, 5.
45. Neave (1939), J. Dairy Res., **10**, 475.
46. Herschdörfer & Neave (1937), J. Soc. chem. Ind., **56**, 781.
47. Gilcreas & Davis (1937), Proc. int. Ass. Milk Dlrs., Lab. Sect., **30**, 34.
48. Boynton & Nelbach (1938), J. Milk Tech., **1** (4), 8.

49. Gibbs (1927), J. biol. Chem., **72**, 649.
50. Scharer (1938), J. Milk Tech., **1** (5), 35.
51. Scharer (1938), Proc. int. Ass. Ice Cream Mfrs., **38**, 2, 50.
52. Scharer (1939), J. Milk Tech., **2**, 16.
53. Tracy & Hahn (1937), Proc. int. Ass. Milk Dlrs., Lab. Sect., **30**, 57.
54. Hahn & Tracy (1939), J. Dairy Sci., **22**, 191.
55. Aschaffenburg & Neave (1939), J. Dairy Res., **10**, 485.
56. Roger (1938), Amer. J. publ. Hlth., **28**, 1325 ; (1939), J. Milk Tech., **2**, 21.
57. Leahy (1938), J. Bact., **36**, 670.
58. Leahy (1939), J. Milk Tech., **2**, 147.
59. Jacobsen (1936), Z. Untersuch. Lebensmitt., **71**, 515.
60. Trembath & Newman (1938), J. Dep. Agric. Vict., **36**, 88.
61. Fay (1939), Milk Pl. Mon., **28** (4), 23.
62. Moffatt & Mackay (1938), Canad. publ. Hlth J., **29**, 283.
63. Tiedeman (1938), Amer. J. publ. Hlth, **28**, 316.
64. Pincus (1938), Amer. J. publ. Hlth, **28**, 322.
65. Kay & Neave (1935), Lancet, **228**, 1516.
66. Hoy & Neave (1937), Lancet, **233**, 595.
67. Smith (1937), Canad. Dairy Ice Cr. J., **16** (9), 26.
68. Tiedeman & Hohl (1938), Amer. J. publ. Hlth, **28**, 629.
69. Milligan (1936), Med. Offr., **55**, 87.
70. Krueger (1939), Amer. J. publ. Hlth, **29**, 477.
71. Krueger (1938), J. Milk Tech., **1** (7), 29.
72. Mattick & Hiscox (1937), XI Milch. Weltkong. Berlin, III, 344.
73. Mattick & Hiscox (1939), Med. Offr, **61**, 177.
74. Winther (1938), Maanedsskr. Dyrlaeg., **50**, 441.
75. (1938–1939), Beretn. Stat. Forsøgsmejeri, Hillerød, No. 16, 17, 18, 20.
76. Parfitt & Brown (1939), Nat. Butt. Cheese J., **30** (1), 12.
77. Mattick (1939), Chem. Ind., **58**, 70.
78. Shadwick & Parker (1939), Amer. J. publ. Hlth, **29**, 482.
79. Hahn & Tracy (1939), J. Dairy Sci., **22**, 219.
80. Caulfield & Martin (1939), J. Dairy Sci., **22**, 261.
81. American Public Health Association (1939), Standard methods for the examination of dairy products (7th ed.). New York : The author.
82. (1935) Rep. med. Offr. Minist. Hlth, Lond., p. 137.
83. (1936) Rep. med. Offr. Minist. Hlth, Lond., p. 144.
84. (1938–39) Yearb. Amer. publ. Hlth Ass., **9**, 36.
85. Gilcreas (1939), J. Ass. off. agric. Chem., Wash., **22**, 497.

NOTES

C. F. T. R. I

POYNDER & SON READING ENGLAND

C.

Acc. No. 5

Call No. F8

CHECKED
2008

VERIFIED
2013

CFTRI-MYSORE
503
Phosphatase test

Acc. No. 503
3C:6;8 N39
(HD)
atase test
efficiency
ation